BIOTECHNOLOGICAL MODIFICATION OF STEROIDAL STRUCTURES

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

UMUT ERKILIÇ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

FEBRUARY 2008

Approval of the Thesis:

BIOTECHNOLOGICAL MODIFICATION OF STEROIDAL STRUCTURES

submitted by **UMUT ERKILIÇ** in partial fulfillment of the requirements for the degree of **Master in Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Gülay Özcengiz
Head of Department, **Biotechnology** _____

Prof. Dr. Ayhan Sıtkı Demir
Supervisor, **Chemistry Department, METU** _____

Prof. Dr. Zümrüt B. Ögel
Co-Supervisor, **Food Engineering Dept., METU** _____

Examining Committee Members:

Prof. Dr. Haluk Hamamcı
Chemistry Department, METU _____

Prof. Dr. Ayhan Sıtkı Demir
Food Engineering Dept., METU _____

Prof. Dr. Gülay Özcengiz
Biology Department, METU _____

Prof. Dr. Pınar Çalık
Chemical Engineering Dept., METU _____

Assoc. Prof. Dr. Nezire Saygılı
Faculty of Pharmacy, HU _____

Date: 05.02.2008

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name : Umut ERKILIÇ

Signature :

ABSTRACT

BIOTECHNOLOGICAL MODIFICATION OF STEROIDAL STRUCTURES

Erkılıç, Umut
M.S., Department of Biotechnology
Supervisor: Prof. Dr. Ayhan Sıtkı Demir
Co-supervisor: Prof Dr. Zümrüt B. Ögel

February 2008, 71 pages

Steroids are important biological regulators existing in hormones which are used to control metabolism of the body. There are widespread applications in the pharmaceutical industry. Drugs of steroid nature - anti-inflammatory and anti-allergic corticosteroids, diuretics, anabolics, androgens, gestagens, contraceptives, antitumor medications, etc. - are now widely used in human and veterinary medicine.

Nowadays, biotechnological modifications of steroids are preferred over chemical modifications as a green chemistry since they are more likely to be natural.

In this work four different *Fusarium* species were screened for bioconversion of steroids into pharmaceutically important derivatives of steroids by reduction, dehydrogenation, side-chain degradation etc. on A and D-rings containing many active sites.

Fusarium spp. used in this work, namely *Fusarium roseum* OUT 4019, *Fusarium anguioides* OUT 4017, *Fusarium bulbigenum* OUT 4115 and *Fusarium solani* OUT

4021 are filamentous fungi, which belong to the class of Deuteromyces. They can grow using simple carbohydrates and nitrogen sources.

4-androstene-3,17-dione conversion is used as a model system. Under same environmental conditions it is found that whole cells of *Fusarium roseum* OUT 4019 can dehydrogenate at C-1 and C-2 producing androsta-1,4-diene-3,17-dione and also reduce at C-17 in addition to dehydrogenate at C-1 and C-2 producing 17-hydroxy-androsta-1,4-dien-3-one, *Fusarium anguioides* OUT 4017 can reduce at C-17 producing 17-hydroxy-androst-4-en-3-one, *Fusarium solani* OUT 4021 can reduce at C-3 and C-17 producing androst-4-ene-3,17-diol at 25 C° and 160 rpm with uncontrolled pH.

In these conversions, androsta-1,4-diene-3,17-dione, 17-hydroxy-androsta-1,4-dien-3-one, 17-hydroxy-androst-4-en-3-one, androst-4-ene-3,17-diol were isolated with 54 %, 22 %, 26 %, 90 % yields, respectively.

In another study, bioconversion reactions of aromatic methyl ethers by *Fusarium roseum* OUT 4019 were investigated and for some compounds, cleavage of methyl ether was observed.

Keywords: 4-androstene-3,17-dione, *Fusarium* spp., C-3 and C-17 Steroidal Ketone Reduction, Steroidal dehydrogenation, Ether Cleavage.

ÖZ

STEROİT YAPILARIN BİYOTEKNOLOJİK MODİFİKASYONU

Erkılıç, Umut
Yüksek Lisans, Biyoteknoloji Bölümü
Tez Yöneticisi: Prof. Dr. Ayhan Sıtkı Demir
Yardımcı Tez Yöneticisi: Prof. Dr. Zümrüt B. Ögel

Şubat 2008, 71 sayfa

Steroitler vücut metabolizmasının denetiminde kullanılan hormonların yapısında bulunan önemli biyolojik düzenleyicilerdir. İlaç sanayinde yaygın uygulamaları bulunmaktadır. Doğal steroit ilaçlar: anti-inflammatör ve anti-allerjik kortikosteroitler, diüretikler, doğum kontrol ilaçları, tümör tedavi ilaçları, vücut geliştiriciler v.b. birçok özelliklerinden dolayı tıp ve veterinerlikte yaygın olarak kullanılmaktadır.

Günümüzde, steroitlerin biyoteknolojik modifikasyonları doğal olmaya daha yatkın oldukları için bir yeşil kimya olarak kimyasal modifikasyonların yerine kullanılmaktadır.

Bu çalışmada dört değişik mikroorganizma türü A ve D halkalarında birçok etkin bölge bulunan steroitlerin önemli ilaç türevlerine indirgenme, dehidrojenasyon, yan zincir parçalanması v.b. yöntemlerle dönüşümleri için taranmıştır.

Bu çalışmada kullanılan *Fusarium roseum* OUT 4019, *Fusarium anguioides* OUT 4017, *Fusarium bulbigenum* OUT 4115 ve *Fusarium solani* OUT 4021 türlerini

içeren *Fusarium* cinsi Deuteromyces sınıfına ait olan filamentus fungi çeşididir ve çok basit karbon ve azot kaynakları kullanarak kolayca çoğalabilmektedirler.

4-androsten-3,17-dion'un dönüştürülmesi model sistem olarak kullanılmıştır. Aynı çevresel koşullardaki hücre türlerinden *Fusarium roseum* OUT 4019, C-1 ve C-2 bölgesinde dehidrojenasyon reaksiyonlarıyla androsta-1,4-dien-3,17-dion ürününü ve de aynı bölgede çift bağ oluşumunun yanı sıra C-17'de ketonun indirgenmesi ile 17-hidroksi-androsta-1,4-dien-3-on ürününü oluşturmuştur. *Fusarium anguioides* OUT 4017 C-17 ketonun indirgenmesi ile 17-hidroksi-androst-4-en-3-on ürününü, *Fusarium solani* OUT 4021 ise C-3 and C-17 ketonların indirgenmesi ile androst-4-en-3,17-diol ürünü 25 °C'de ve 160 devir/dakika ve kontrolsüz pH ortamında oluşturmuştur.

Bu dönüşüm tepkimelerinde, androsta-1,4-dien-3,17-dion, 17-hidroksi-androsta-1,4-dien-3-on, 17-hidroksi-androst-4-en-3-on, androst-4-ene-3,17-diol ürünleri sırasıyla 54 %, 22 %, 26 %, 90 % verimlerde elde edilmiştir.

Diger çalışmada, aromatik halkaya bağlı metil eter içeren bileşiklerin *Fusarium roseum* OUT 4019 ile biyodönüşümleri incelenmiş ve bazı örneklerde methy eter kısmının koparılarak alkole dönüştürüldüğü saptanmıştır.

Anahtar kelimeler: 4-androsten–3,17-dion, *Fusarium*, C-3 ve C-17 Steroidal Ketonların İndirgenmesi, Steroidal Dehidrojenasyon, Eter Bağı Koparma.

To my father and mother

ACKNOWLEDGEMENT

I owe my special thanks and sincere appreciation to Prof. Dr. Ayhan Sıtkı Demir for his invaluable guidance, supervision, encouragement, insight and understanding throughout the research.

I would like to express my deepest thanks to my family for helping, supporting, encouraging and loving me all through my life.

I would like to express my special thanks to my friends Peruze Ayhan, Ş. Betül Sopacı, Hacı Eşiyok, Umut Demirtaş and İlke Şimşek for their helps and friendships during this research.

I am also indebted to Mrs. Fatoş Doğanel Polat and Ms. Seda Karayılan for their technical support.

Finally, I would also like to thank all of the members of Ayhan Sıtkı Demir research group for their help, friendship, and cooperation.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

CHAPTER 1

INTRODUCTION

1.1. Bioconversion in organic chemistry

Bioconversion is the conversion of chemicals into usable products or energy sources by biological processes or agents, such as certain microorganisms or enzymes **[1]**.

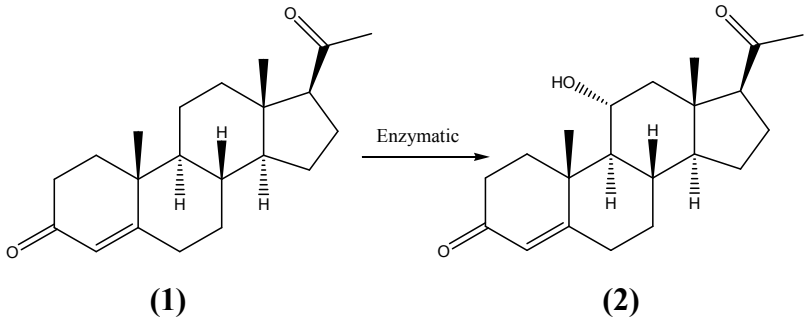
The development of scientific screening and isolation methods allows the selection of desirable natural occurring or mutating microorganisms for specific purposes. The diverse catalytic activities of microorganisms are being used more and more widely to perform specific chemical reactions **[2]**.

Apart from the biosynthesis and biodegradations, the enzymes of microorganisms have been used for decades for biotransformations. These reactions are transformations of individual educts in identifiable type reactions resulting in metabolites with chemically defined structures. Biotransformations also represent a useful tool for organic chemistry because of their main advantages: attack of non-activated positions, reaction specificity, region-, stereo- and enantioselectivity and the mild reaction conditions **[3]**.

These advantages are obvious in the special modification of natural or chemically synthesized compound by selective enzymatic reactions for the preparation of pharmaceuticals, agrochemicals, food constituents, fine chemicals, even bulk chemicals. The general targets are: the shortening of chemical multistep synthesis, elimination of side reactions, preparation of optically active synthons, so-called chirons, the imitation of mammalian metabolism, the preparation of "natural"

products instead of compounds identical to natural ones and using the ecological profits: saving energy and decrease of environmental pollution compared to chemical procedures **[3]**.

Enantiopure compounds have undoubtedly gained a central role in the development of chemical technology. This is most evident from the changes in the drug market, where single-enantiomer drugs currently occupy the highest share. The request for more efficient, more specifically targeted drugs will place a growing demand on enantiopure materials. The first industrially applied microbiological fermentation, paving the way to the development of the subject area of the present treatise, was the introduction of the 11-hydroxy group into a progesterone nucleus, achieved by the Upjohn Comp. in 1951. Thus, 11α-hydroxy-progesterone could be transformed into cortisone in nine steps, allowing the synthesis of cortisone in only 14 steps form diosgenin **[4]**.



(1) **(2)**

Figure 1. Enzymatic reaction of progesterone **(1)** into 11α-hydroxy-progesterone **(2)**

Numerous reaction types can be used and the application of biotransformations in organic chemistry is sometimes named "chemical reactions with microorganisms and enzymes" **[3]**.

A version of biotransformation is direct synthesis. This is feeding an organism with a chemical that would not normally be present in its environment so that its normal metabolic pathways include that chemical into a new product **[5]**. Another version is

indirect synthesis. In this method, chemical material isolated from the environment of the organism is given to it so that its metabolism convert the material to the desired product, or by using enzyme of it in a suitable condition imitating the metabolism of the organism specific product is produced.

The most commonly used biotransformations involve acylases, esterases, lipases, beta lactamases, penicillin acylase, peptidases, proteases, and *steroid transforming enzymes*. These are always used in whole organisms, as many enzymes are involved in each biotransformation. There are very wide ranges of microorganisms used in biotechnology. Some are used for more than one thing and so crop up in several biotechnological contexts. Some of the more commonly used organisms are *Aspergillus niger, Bacillus subtilis, Candida utilis, Clostridium acetobutylicum, Corynebacterium glutamicum, Escherichia coli, Penicillium, Pischia pastoris, Pseudomonas, Saccharomyces and Streptomycetes* **[5]**.

The areas of microbial biotechnology that are now receiving attention are: application of the newer concepts of genetic engineering of microorganisms for their improvement as transforming agents; solubility improvement for carrying out biotransformation of substrates that are sparingly soluble in water; immobilization of enzymes or whole cells in a suitable matrix for repetitive economic utilization of enzymes; development of a continuous process for economic product recovery; and manipulation of culture media for improvement in product yields **[6]**.

1.1.1. Fungi mediated bioconversions

Fungal biotransformation of steroids is among the earliest examples of biocatalysis for producing stereo- and site-specific products, including the commercially important cytochrome P450- mediated steroid hydroxylation **[7]**.

For many years fungi have been used for the hydroxylation of steroids since they have an ability to catalyze reactions by enzyme systems with high regio- and stereospecifity, which is known as a common feature of filamentous fungi **[8]**.

Since 1950, the range of reactions that can be efficiently carried out by fungal bioconversions has been expanded enormously, and now includes examples of hydrolytic, oxidation, condensation, and reduction processes. Other studies have focused on substrate groups such as steroids, alkaloids, sulfides, environmental pollutants and bioactive compounds; on single bioconversion reactions such as halogen metabolism, and alcohol dehydrogenase activity **[9]**.

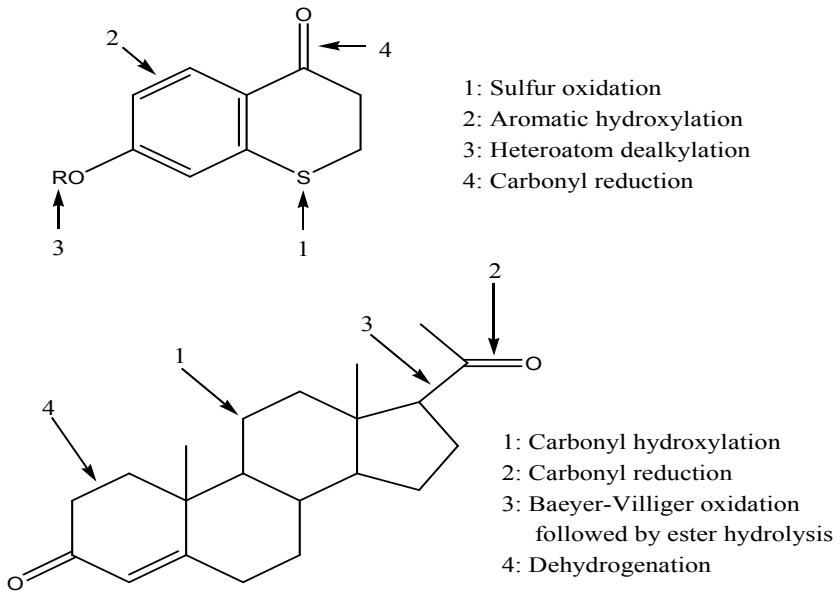


Figure 2. Common reactions of fungal bioconversion **[9]**

The most frequently observed reactions of fungal bioconversions are shown in Figure 2 for two common substrate groups, steroid and aromatic compounds. Not all of these reactions occur with every substrate, but the most common, the hydroxylation and Baeyer-Villiger oxidations. Thus, although it is possible to distinguish, for example, steroid hydroxylation from Baeyer-Villiger oxidation and dehydrogenation by selection of the appropriate fungus for bioconversion, it may not always be possible to select fungi that will hydroxylate a steroid ester without some degree of ester hydrolysis, or sulfoxidize a keto sulfide without reduction of carbonyl group **[9]**.

1.1.2 *Fusarium* species in bioconversion reactions

Fusarium is a type of fungi either pathogen or producing toxin or both and widespread in the soil. As fungi with different strains, they perform many chemical reactions with their enzymes. Transformation of cyclohexanone **(3)** to ε-caprolactone **(4) [10]**, degradation of benzopyrene **(5) [11]**, reduction of NO molecules with nitric oxide reductase **[12]**, conversion of furostanol glycoside precursor of diosgenin into diosgenin with very high efficiency **[13]**, regio- and stereoselective hydroxylation of bi- and tricyclic enones **(6),(7) [14]**, degradation of phenol **[15]**, transformation of ginsenoside Rg_3 to Rh_2, which is a very potent antitumor agent **[16]** are some examples of bioconversions of *Fusarium* species.

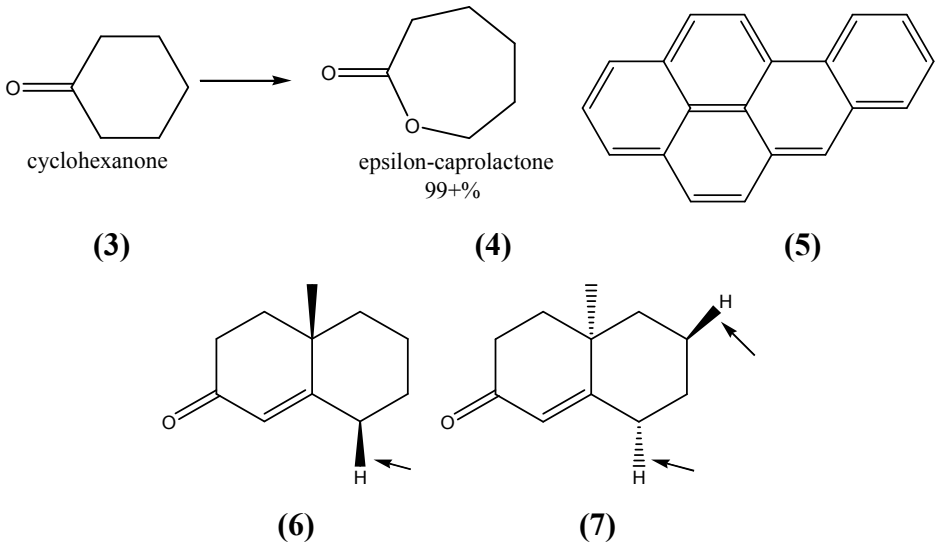


Figure 3. *Fusarium* species catalyzed reactions

Moreover, *Fusarium* genus of the fungi has an ability to hydroxylate steroidal substrates: some *Fusarium* strains are good hydroxylators at the 11α position of Reichstein's substance. The 15α-hydroxyl group was introduced to hormones: testosterone, androstenedione, progesterone and estrone by all the *Fusarium* strains able to hydroxylate these substrates. Quite a large number of these strains converted androstenedione, testosterone to a mixture of 6β- and 15α- hydroxyl derivatives **[17]**.

1.2. Steroids

A steroid is a terpenoid lipid characterized by a carbon skeleton with four fused rings, generally arranged in a 6-6-6-5 fashion **[18]**.

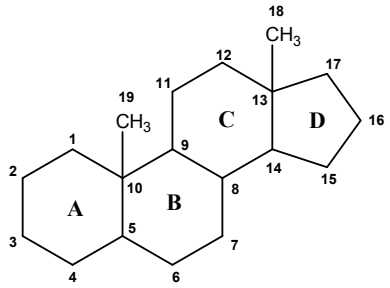


Figure 4. Steroid skeleton structure

Steroids vary by the functional groups attached to these rings and the oxidation state of the rings. Hundreds of distinct steroids are found in plants, animals, and fungi. All steroids are made in cells either from the sterol lanosterol (animals and fungi) or the sterol cycloartenol (plants). Both sterols are derived from the cyclization of the triterpene squalene **[18]**.

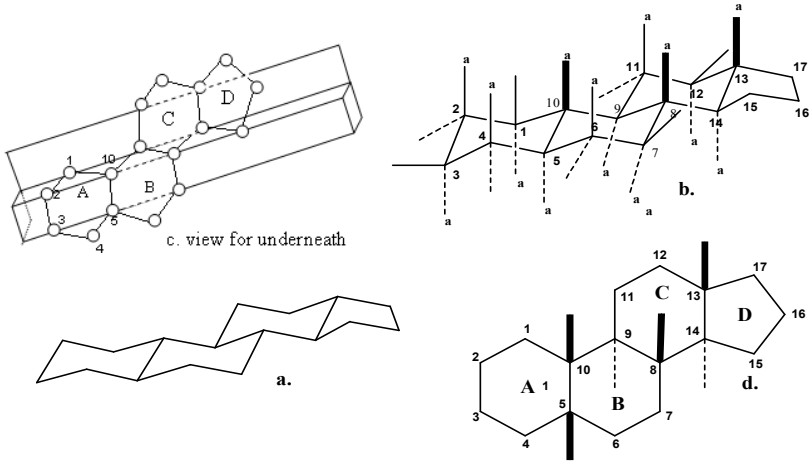


Figure 5. 5α (Allo) series, rings A/B *trans* **[19]**

The steroid nucleus is a rather rigid, essentially planar structure with substituents above the plane of the rings designated ***β*** (solid line) and those below called ***α*** (dotted line). Most steroids in humans have methyl groups at position 10 and 13 frequently a

side chain at position 17, which is β oriented, also most of them are non-polar **[66]**. The principal ones are cholestane (all 27 C's), cholane (C's 1-24), pregnane (C's 1-21), androstane (C's 1-19) and oestrane (C's 1-18) **[21]**.

In most steroids the B, C and C, D ring junctions are *trans* **[22]** and they are both locked in the chair conformation. The junction between rings A and B can be either *trans*, giving the 5α or "allo" series of compounds, or *cis*, giving the 5β or "normal" series of compounds **[19]**.

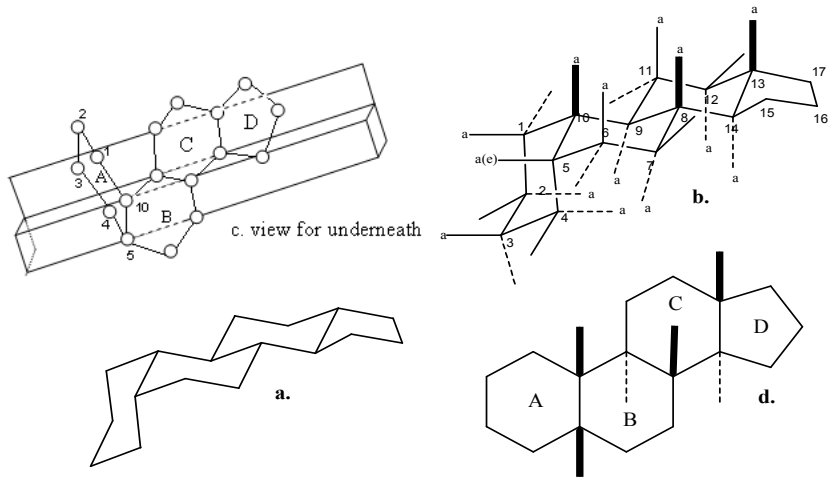


Figure 6. 5β (Allo) series, rings A/B **[19]**

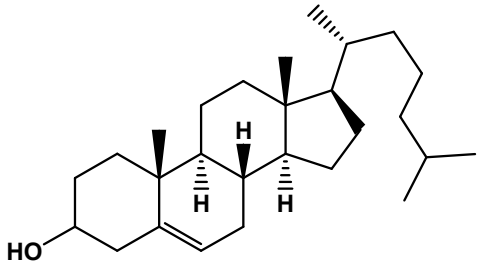
Most of the biologically active steroids have a *trans* junction between rings A and B and belong to the 5α or "allo" series. This makes the 4-rings almost completely planar (as shown in **Figure 4.**), which represents four different ways of showing the same molecule. **Figure 4.a** shows a sideways view of the four rings without hydrogen bonds, **Figure 4.b** shows the same view with the position of the hydrogen bonds. **Figure 4.c** shows a three dimensional picture of the carbon skeleton, **Figure 4.d** shows the conventional drawing of the basic structure **[19]**. In systematic nomenclature, the nature of the R group at position 17 determines (primarily) the base name of an individual steroid **[22]**.

1.2.1. Important steroid types, their structures and biological activities

In the categories of steroids, three main groups exist: animal, plant and fungus steroids. Animal steroids composed of vertebrate steroids containing steroid hormones and cholesterol. For steroid hormones, they have mainly sex steroids (androgens, estrogens and progestagens), corticosteroids (glucocorticoids, mineralocorticoids), and also anabolic steroids **[18]**.

Steroids occupy an important position among pharmaceutical preparations used for treating and preventing diseases of various groups in endocrinology, oncology, rheumatology, gynecology, etc. The relatively broad nomenclature of efficient steroid drugs is continually expanding. Of note, several preparations administered for life-saving indications have no non-steroid analogues **[23]**.

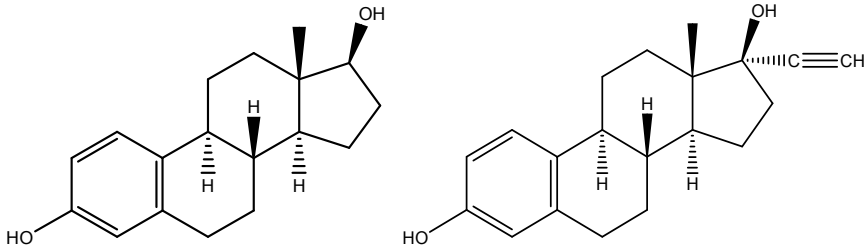
Most steroid hormones are active in extremely minute amounts **[19]**. These small, relatively similar molecules are able to have greatly differing effects because the slight structural differences among them allow interactions with specific receptor molecules **[24]**. They still play an important role in the drug industry. Drugs of steroid nature - anti-inflammatory and anti-allergic corticosteroids, diuretics, anabolics, androgens, gestagens, contraceptives, antitumor medications, etc. - are now widely used in medicine and veterinary medicine **[25]**. The hormone preparations make up the widest group of these drugs, which are mainly used as contraceptives **[26]**. There are many types of steroid but cholesterol, estradiol, progesterone, testosterone, aldosterone and cortisol are the most important ones.



(8)

Figure 7. Cholesterol

Cholesterol **(8)** is the only steroid which is plentiful in vertebrates, but it appears not to have hormonal activity **[21]**. It occurs widely in human body as a component of cell membranes. It keeps the fluidity of cell membranes at the right level **[27]** but not all of the biological functions of cholesterol are yet known. Cholesterol is known as a serve as an intermediate in the biosynthesis of all steroids of the body which is called steroid hormones. Among them are estrogen, testosterone, and cortisol **[22]**. These hormones are powerful signal molecules that regulate a host of organismal functions; therefore it is essential for life **[24]**.



(9) **(10)**

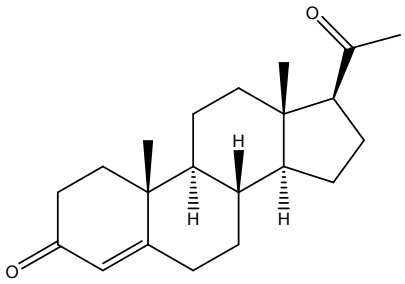
Figure 8. Estradiol and Ethynylestradiol

Estradiol **(9)**, which is the principle female sex hormones, is secreted by the ovaries and promotes the development of the secondary female characteristics that appear at the onset of puberty. Estrogens also stimulate the development of the mammary glands during pregnancy and induce estrus (heat) in animals **[22]** and also participate in the control of the menstrual cycle **[28]**.

Synthetic estrogens have also been developed and these are often used in oral contraceptives in combination with synthetic progestins. A very potent synthetic estrogen is the compound called *ethynylestradiol* or *novestrol* **(10)** **[22]**. Other medicinal uses for estrogens are the treatment of estrogen deficiency, prostate cancer, and suppression of lactation **[21]**.

Progesterone **(11)** is the most important pregnancy hormone. It interacts strongly with estrogens in its effects on tissues **[19]**. This hormone prepares the lining of the

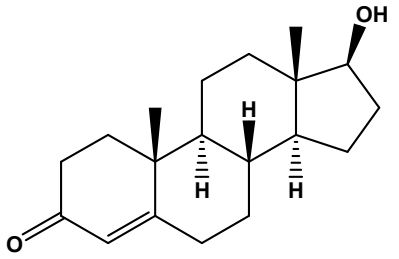
uterus for implantation of the fertilized ovum, and continued progesterone secretion is necessary for the completion of pregnancy **[22]**.



(11)

Figure 9. Progesterone

Progesterone also suppresses ovulation, and it is the chemical agent that apparently accounts for the fact that pregnant women do not conceive again while pregnant. Therefore, with this function, synthetic progesterone could be used as oral contraceptives. Progesterone, itself, requires very large doses to be effective in suppressing ovulation when taken orally because it is degraded in the intestinal tract. A number of such compounds have been developed and are now widely used **[22]**.



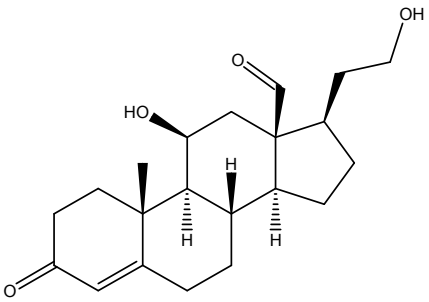
(12)

Figure 10. Testosterone

Testosterone **(12)**, which is one of the male sex hormones secreted by the testes, is the hormone that promotes the development of secondary male characteristics; the growth of facial and body hair; the deepening of the voice; muscular development; and the maturation of the male sex organs **[22]**, and so a deficiency may lead to a low sperm count and impotence **[21]**.

Androgens are used in the treatment of male sterility, impotency and female breast and genital cancers **[26]**. The conversion of androgens to estrogens is catalyzed by aromatase and so Numazawa *et al.* states that 2-methyleneandrostenedione is a powerful inhibitor of aromatase which is useful in treating estrogen-dependent breast cancer **[29]**. Anabolic drugs having low androgenicity are useful in the treatment of underdeveloped children and for patients having debilitating diseases or in convalescence **[21]**. In addition, synthetic testosterone analogs are used in medicine to promote muscle and tissue growth, i.e. in patients with muscular atrophy **[28]**.

Corticosteroids are divided into two main groups depending on the biological activity. The mineralocorticoids affect the excretion of fluid and electrolytes. The glucocorticoids affect intermediary metabolism and suppress inflammatory processes **[19]**.



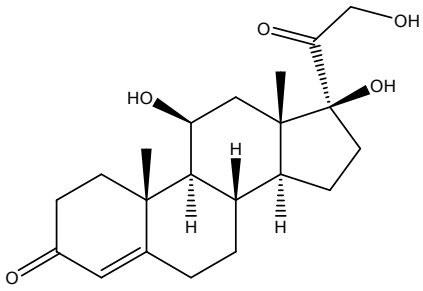
(13)

Figure 11. Aldosterone

Aldosterone **(13)** is the principle mineralocorticoid, produced from progesterone in adrenal cortex and it raises blood pressure and fluid volume **[30]**, controls the sodium potassium levels **[21]**.

Cortisol **(14)** is dominant glucocorticoid in humans, synthesized from progesterone in adrenal cortex and it is involved in stress adaptation, elevates blood pressure and Na^+ uptake, and also has numerous effects on immune system **[30]**. In addition, glucocorticoids promote gluconeogenesis and formation of glycogen, enhance

degradation of fat and protein, and inhibit the inflammatory response. They enable animals to respond to stress- indeed, the absence of glucocorticoids can be fatal **[24]**.



(14)

Figure 12. Cortisol

Most of the adrenocortical steroids have an oxygen function at position 11 (a keto group, β-hydroxyl, in cortisone). The adrenocortical steroids are apparently involved in the regulation of biological activities including carbohydrate, protein and lipid metabolism (glucocorticoids), water and electrolyte balance, and reactions to allergic and inflammatory phenomena (mineralocorticoids). By the recognition of anti-inflammatory effect of cortisone and its usefulness in the treatment of rheumatoid arthritis, cortisone has been studied extensively. Many 11-oxygenated steroids are now used in the treatment of a variety of disorders ranging from Addison's disease to asthma and to skin inflammations **[22]**.

Fokine *et al.* states that dehydrogenated derivatives of corticosteroids are usually more effective than their precursors in treating such diseases as rheumatism, unspecific infectious polyarthritis and bronchial asthma **[31]**.

Another type of steroids is the plant steroids. Plant steroids are classified into sterols, cardiac glycosides, saponins, and alkaloids. The major function of plant sterols is thought to be, as in animals, to act as a building block in membranes. Plant steroidal glycosides which is also called as cardiac glycosides, have a powerful action on heart muscle **[21]**.

1.2.2. Chemical properties of steroids

Steroids are generally stable, crystalline compounds with similar properties to those of simpler analogues except that their reactions tend to be highly stereo- and regio- selective. As an aspect of regioselectivity, the reactivities of steroidal functional groups depend on their positions. The commonly encountered positions of steroidal carbonyl groups are 3, 11, 17 and 20 and their reactivities towards nucleophilic attack (e.g. hydride reduction, ketal formation, and Grignard addition) are in the order 3 > 17 ≥ 20 > 11, which is also the order of increased steric crowding. For stereoselectivity, many steroid reactions may yield two or more products differing in the orientation (α or β) of the groups participating in the reaction, and the predominant isomer may alter with the reaction conditions **[21]**.

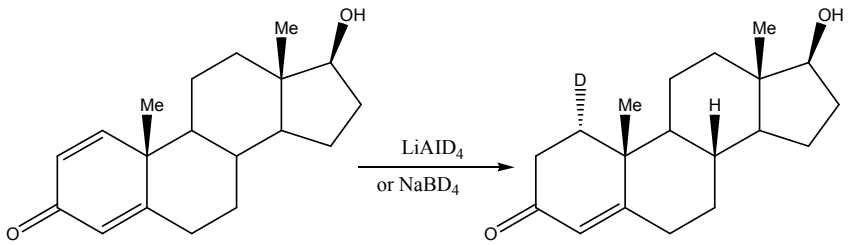
1.2.3. Chemical conversion reactions with steroids

Steroids undergo all of the reactions that we might expect of molecules containing double bonds, hydroxyl groups, keto groups and so on. While stereochemistry of steroid reactions is often quite complex, it is many times strongly influenced by the steric hindrance presented at the β face of the molecule by the angular methyl groups. Many reagents react preferentially at the relatively unhindered α face, especially when the reaction takes place at a functional group very near an angular methyl group and when the attacking reagent is bulky **[22]**.

Two strategies, both requiring major chemical advances, were followed: the conversion of a readily available steroids into desired hormones (partial synthesis), and the total synthesis of steroidal hormones from simple chemicals **[21]**. In partial synthesis, as the starting materials for the chemical preparation of the various structures, it is basically the natural product deoxycholic acid, stigmasterol and diosgenin **[32]**, sapogenin **[21]** are used.

As a synthetic method, total synthesis is used in the production of steroid structures. Because of their importance in medicinal use steroids have enjoyed a tremendous

amount of synthetic activity, and much of this is already incorporated into the basic organic chemistry texts **[26]**.



(15) **(16)**

Figure 13. Reaction of 17-hydroxy-androst-1,4-dien-3-one with $LiALD_4$ or $NaBD_4$

As an example of recent study performed in steroid conversion by chemical method, reduction of a double bond at C-1 of 17-hydroxy-androst-1-4-dien-3-one **(15)** with $LiAlD_4$ and $NaBD_4$ catalysts gave stereospecifically [1α-D] labeled steroid testosterone **(16)** with 18 % and 15 % yields respectively besides other products **[33]**.

1.2.4. Bioconversion reactions with steroid

Many microorganisms immobilized or not, are used to modify the structure of a broad range of chemicals including antibiotics, alkaloids, cyclic and linear hydrocarbons, pesticides, terpenes and steroids. Steroid transformation, in particular has been a most fruitful area in which microorganisms have been used to alter the structure of chemicals foreign to the microorganism. This application of microorganisms differs conceptually from the most of the other microbial processes with the exception of the foreign protein involved chemicals normally produced by microorganisms. Many microorganisms contain enzymes that recognize steroids as substrates even though they are, in general, novel to the organism. These microbial enzymes recognize particular domains within unfamiliar chemicals, presumably because these domains bear a resemblance to related domains within familiar molecules **[34]**. Therefore, steroid compound can be ranked among the most widely marketed products from the pharmaceutical industry **[35]**.

The importance of microbial biotechnology in the production of steroid drugs and hormones was realized for the first time in 1952 when Murray and Peterson patented the process of 11α-hydroxylation of progesterone by a *Rhizopus* species **[36]**. Therefore, to make 11-hydroxy steroids were available in limited quantities. It had been known that yeast could dehydrogenate and hydrogenate steroids since 1937. However, the substrates used are foreign to the microorganisms and also natural sterols, e.g. ergesterols, are resistant to transformations by means of microorganism. After isolation of other *Rhizopus* species, 11-α hydroxylation product of steroids were obtained with higher yields **[37]**. Previously, this product could only be prepared starting from deoxy cholic acid on via 26 chemical steps **[3]**.

Since then, microbial reactions for the transformation of steroids have proliferated, and specific microbial transformation steps have been incorporated into numerous partial syntheses of new steroids for evaluation as drugs and hormones. These biotransformations have provided adequate tools for the large scale productions of natural or modified steroid analogues. The latter are currently favored when compared to their natural counterparts due to some therapeutic advantages, such as an increased potency, longer half-lives in the blood stream, simpler delivery methods, and reduced side effects. The preferential use of whole cells over enzymes as biocatalysts for the production of these pharmaceutical derivatives mostly results from the costs of the latter enzyme isolation, purification, and stabilization **[36]**. In addition, these biotransformation approaches may involve the use of free or immobilized cells or enzymes both in aq. and organic media (two phase system) **[38]**.

Furthermore, the use of microbial models to mimic mammalian metabolism is well known **[36]**, the reactions of microorganisms performed on steroid compounds have been valuable in the solution of specific problems in steroid metabolism in animals and human body, and in conjunction with chemical syntheses, in the commercial production of useful and complex steroids. Nowadays, in addition to the hydroxylation reactions, at almost any position of steroid nucleus, many organisms have been found performing many bioconversion reactions such as oxidation, dehydrogenation, reduction, and side-chain degradation etc **[37]**.

The oxidation and reduction reactions that microorganisms perform on steroid provide particularly impressive examples of regio-selective and stereo-specific biotransformations and also demonstrate the ability of enzymes to promote reactions at inactivated centers in hydrocarbons **[39]**.

In the production of specific steroids, a precursor steroidal compound is isolated from some biological source such as ox bile, urine, or a plant. Once a precursor is obtained, it is converted into the desired form by using a combination of microbiological and chemical methods. If such reaction is chemically difficult, expensive or otherwise impractical, and if a microbe can accomplish it readily and inexpensively, the transformation is done with the aid of a microbe **[40]**.

One of the main drawbacks in steroid biotransformation is the low solubility of the substrates in water, which diminishes reaction rates and overall productivity **[41]**. Therefore, the utilization of high amounts of the steroidal substrates is one of the important factors affecting the economy of the transformation process **[42]**.

For the conversion of steroids, biotechnological methods involving whole cells or enzymes of the microorganism are primarily preferred due to many advantages when compared to chemical conversion methods.

Microorganisms have been widely applied for biotransformations of steroids in order to prepare derivatives which are difficult to obtain in a different way. The following three microbial steroid modifications are particularly important in modern biotechnology: selective side chain cleavage of natural sterols, 1-dehydrogenation, and 11-hydroxylation **[51]**. These biotransformations, mostly associated to chemical synthesis steps, have provided adequate tools for the large scale production of natural or modified steroid analogues. The manufactured steroid compounds have a wide range of therapeutic purposes, namely, as anti-inflammatory, immunosuppressive, progestational, diuretic, anabolic and contraceptive agents **[35]**.

1.2.4.1. Hydroxylation

Microbial hydroxylations are among the most studied and useful transformations since they can be achieved under mild conditions with high chemo-, regio- and stereoselectivity **[41]**. This procedure also remains one of the most useful preparative methods for the introduction of hydroxyl groups at sites of the steroid nucleus remote from other functionality, and the value of microbial steroid hydroxylation in the preparation of pharmacologically active steroids is well-established **[43]**.

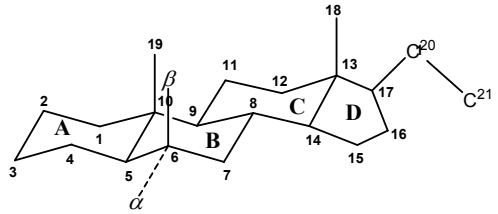
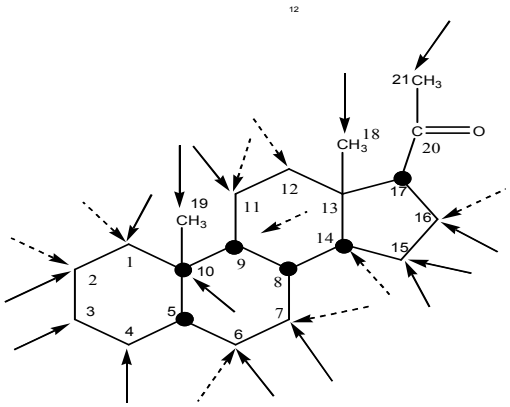


Figure 14. Hydroxylation positions on steroidal structure

Hydroxylations can be used to build intermediates for further chemical synthesis, by offering access to otherwise inaccessible sites of the steroid molecule, or to provide the steroid molecule with the adequate structure for therapeutic applications **[35]**.

Virtually any position in the carbon skeleton of a steroid nucleus can be hydroxylated stereospecifically by enzymes present in some microorganisms. Steroid hydroxylases are named according to the position they attack on the rings or the side chain of the steroid nucleus **[39]**.



1β-position	=	*Botryodiplodia malorum*
2β-position	=	*Gnomonia funicolata*
6β-position	=	*Rhizopus arrhizus*
7α-position	=	*Botryodiplodia theobromae IFO 6469*
7β-position	=	*Botryosphaeria obtusa CBS 38560*
8β-position	=	*Corynespora melonis CBS 16260*
9α-position	=	*Corynespora cassiicola ATCC 16718*
12α-position	=	*Cercospora kaki CBS 12839*
14α-position	=	*Mucor griseocyanus ATCC 1207 a*
1β-position	=	*Penicillium stolonifer CBS P102*
15α-position	=	*Aspergillus fumigatus*
15β-position	=	*Sepedonium ampullosporum*
16α-position	=	*Aspergillus niger NRRL 599*
16β-position	=	*Trichothecium roseum*
17α-position	=	*Corynespora cassicola IMI 56007*
18	=	*Nigrospora sphaerica ATCC 12772*
19	=	*Ophiobolus herpotrichus*

Figure 15. Reactions on steroid skeleton by various fungi species **[3]**

There are three primarily carbon atoms (C_{18}, C_{19}, and C_{21}) and also 18 secondary carbon atoms within the ring system, there are two alternative ways, designated α and β, to attach the –OH group. Every one of the 18 carbon atoms can be hydroxylated, in either α or β configuration, each by a different known microbial hydroxylase **[39]**.

Primary hydroxylations are 11α, 11β, 16α-hydroxylations performed by microorganism. In mammals oxidation of C_{11} is an important transformation of the cortical steroids leading to cortisol and cortisone **[44]**. Transformation of progesterone by using spores of *Aspergillus ochraceus* at 11α- and also 6β, 11α-positions. For 11α-hydroxylaiton of progesterone yield of above 50 % was observed. However, it is stated that it can be increased up to 80-90 % by optimizing growth conditions using fungal spores **[45]**.

Sedlaczek *et al.* applied different method, fungal protoplast, in the transformation of steroids. Using protoplast of *Cunnighamella elegans*, 11α- and 11β-hydroxylation on cortexolone gave better results when compared to that of mycelium: the rate of cortexolone transformation by protoplast is four times higher than that for mycelium **[46]**. Transformation of 2-oxatestosterone gave successful results in the study of Holland *et al.* by *Aspergillus ochraceus* with 82 % isolated yield of 11α-hydroxy-2-oxatestosterone **[43]**. Furthermore, by another *Aspergillus* species, *Aspergillus ochraceus*, hydroxylation of progesterone into 11α-hydroxyprogesterone successfully achieved with approximately 90 % yield **[47]**.

Berrie *et al.* reported that transformation of progesterone into 16α-hydroxyporgesterone achieved with a 1:3.6 ratio to the main product by *Streptomyces reseochromogenes* **[35]**. Transformation of 9α-fluorohydrocortisone into 16α-hydroxylated product was also performed by *Streptomyces* species **[32]**. In addition, it was reported that testosterone, deoxycorticosterone, estrone, estradiol, cortisol, androst-4-ene-3,17-dione etc. were also hydroxylated at C-16α position with *Streptomyces* spp. **[37]**.

7α-hydroxy steroids might play a key role in the regulation of glucocorticoids action and the immune process **[48]**. In the study of Cotillon *et al.,* transformation of 3-hydroxy steroid performed by the DHEA-induced 7α-hydroxylase of *Fusarium moniliforme*. Dehydroepiandrosterone (DHEA) was converted to 7α-hydroxlated product successfully with a yield of 98 % when compared to non-induced yield about 50 % **[48]**.

Moreover, *Botrytis cinera* was found to be an efficient 7α-hydroxylator of steroidal 4-ene-3-ketones. The amount of hydroxy derivatives comprised about 26-82 % of total metabolites for testosterone derivatives. 1-dehydrotestosterone was also significantly hydroxylated at a 14α-position **[49]**. Testosterone derivatives were also converted by *Absidia glauca* into products of 6β, 7α, 7β, 10β, 11α, and 12β or 15β hydroxylation with reasonable yields **[50]**.

Microbial hydroxylation of 2-oxotestosterone was performed by organisms that are known to be efficient hydroxylators at 6β and 15β: at 15β position with 35 % yield by *Bacillus megaterium*, 6β- hydroxy derivative of it with 59 % yield by *Rhizopus arrhizus*, and also 14α-hydroxylation with 40 % yield by *Curvularia lunata* **[43]**.

1.2.4.2. $\Delta^{1,2}$-Dehydrogenation

$\Delta^{1,2}$-dehydrogenation steps remain the most important in microbial transformation biotechnology of the steroid **[42]**. Microbial-$\Delta^{1,2}$-dehydrogenation is usually performed with whole cells **[35]**.

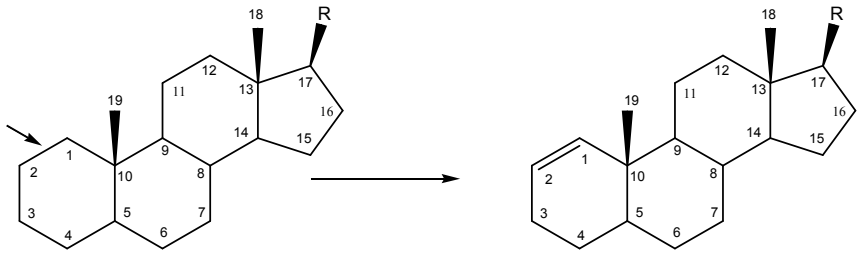


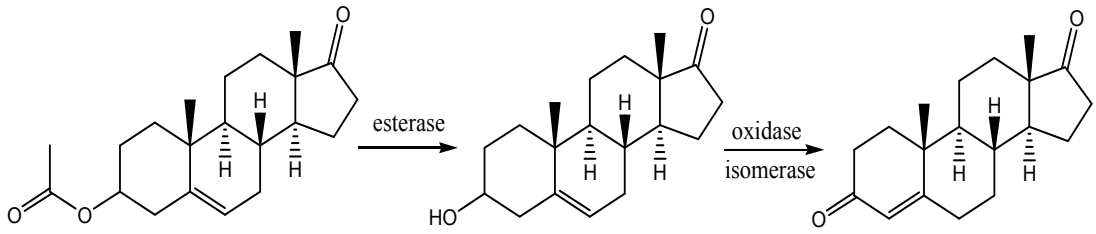
Figure 16. Reaction scheme of $\Delta^{1,2}$-dehydrogenation of steroids

Dehydrogenases are widely spread in bacterial genera on the contrary to fungus. *Arthrobacter*, *Clostridium*, *Corynebacterium*, *Bacillus*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, and *Streptomyces* are some of the species performed this reaction. $\Delta^{1,2}$-dehydrogenation is usually not accompanied by hydroxylation, more often it is the forerunner in a complex series of degradative reactions that ultimately ends in ring cleavage and the complete mineralization of the steroid skeleton **[51]**.

In the transformation of 3-oxosteroids, Bartmanska *et al.* performed mainly $\Delta^{1,2}$-dehydrogenation which has been rarely observed in fungi cultures. In this transformation (conversion on A-ring) cortexolone was converted into 1-dehydrocortexolone by *Trichoderma hamatum* with 89 % yield. Besides this, 6α, 11α, 12β positions, ester bond hydrolysis, oxidation of 17α-hydroxly group, C17/C20 bond scission and ring D-lactonization. It was also stated that absence of 19-methyl group led to lower yield in hydroxylation and 1-dehydrogenation **[51]**.

Songtao *et al.* were used *Arthrobacter simplex* in the conversion of methyl-testosterone into methandienone with a conversion more than 95 % in biphasic system which was used to increase the solubility of product **[52]**. The same reaction was performed with the same species for high concentration of 16-methyl-Reichstein's compound S-21 acetate (16MRSA) in microemulsion system with a conversion of 98 % **[53]** and in the conversion of hydrocortisone to prednisolone at high concentration by immobilized *Corynebacterium simplex* with a conversion of 80 % **[54]**. In another study, the same reaction was performed by *Bacillus sphaericus* ATCC 13805, *Bacillus sphaericus* SRP III and *Arthrobacter simplex* 6946 and 87.6, 70.6, 88.3 % product yields obtained respectively in a two-liquid-phase system using butyl acetate as an organic phase **[55]**.

1.2.4.3. Ester saponification and oxidation of hydroxyl groups



(17) **(18)**

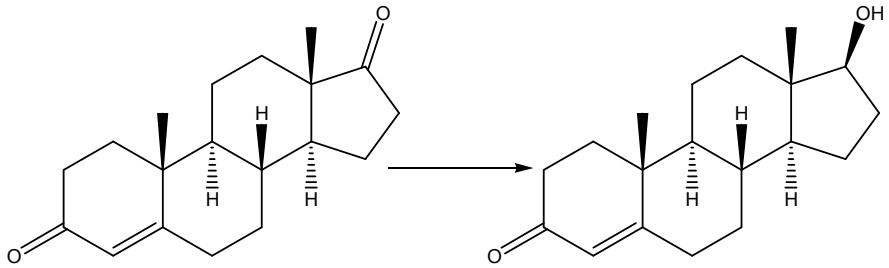
Figure 17. The conversion catalyzed by *Flavobacterium dehydrogenans*

Boeren *et al.* studied *Flavobacterium dehydrogenans* in the transformation of androstenelone-acetate **(17)** into 4-androstene-3,17-dione **(18)** in two-liquid-phase

system with more than obtaining 98% conversion using octane as a second phase in the media **[56]**.

It was reported that *Mycobacterium* species was used for the conversion of phytosterols, β-sitosterol into 4-androstene-3,17-dione **(18)** with a 90 % molar yields **[35]**.

1.2.4.4. Reduction of keto group



(18) **(12)**

Androst-4-ene-3, 17-dione Testosterone

Figure 18. Reaction scheme of ketone reduction

This process is used technically today only on the small scale for the production of testosterone from androst-4-ene-3, 17-dione **[32]**. *Schizosaccharomyces pombe* was used in the transformation of 4-androstene-3,17-dione into testosterone with 95 % conversion **[7]**. The same conversion was performed by immobilized *Arthrobacter simplex* with the conversion of above 50 % using liposomal media to eliminate the solubility problem of steroid at high concentration having an inhibitory effect for microorganisms during bioconversion **[57]**.

1.2.4.5. Sterol side chain degradation

Microbial degradation of the side chain of natural sterols has received much attention in the pharmaceutical industry. The steroidal intermediates from mentioned reaction can be used for the synthesis of all type I aromatase inhibitors and other several high-

value steroidal drugs. The vast majority of microbial side-chain degradation reaction is still based mainly on cholestane-based compounds of both animal and plant origin, such as cholesterol and phytosterol mixtures, sitosterols **[58]**.

In addition, the selective sterol side-chain cleavage is much more limited in both bacteria and fungi being confined mainly to the various species of *Mycobacterium* genus **[58]**. Moreover, the only way to produce AD on a commercial scale is microbiological synthesis, which involves selective cleavage of the side chain of animal (cholesterol) or plant (mainly sitosterol) β-sterols by mutant bacterial strains, which belongs to *Mycobacteria* displaying a high oxidizing activity combined with the ability to emulsify hydrophobic hydrocarbons in aqueous media **[25]**.

Sripalakit *et al.* used different bacterial and fungal strains in the conversion of sterols into various steroids: β-sitosterol was highly converted into total androstenones in yield of 75.87 and 83.86 % by *Mycobacterium* spp. NRRL B-3683 and NRRL B-3805, respectively. Almost equivalent of maximum 4-androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) with total conversion of 81.83 % was observed in mixed cultures of both strains. The molecular structure of natural sterol affected on the productivity of AD and ADD using free strains of those **[58]**.

1.2.4.6. Other types of reactions in microbial transformation of steroid

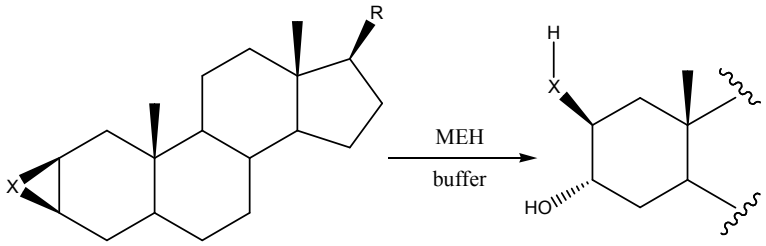


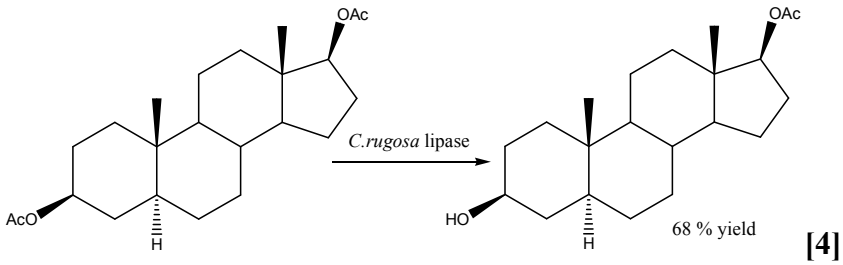
Figure 19. Enzymatic hydrolysis of steroid epoxides

MEH (microsomal epoxide hydrolase) has an ability to hydroxylate the epoxide (fast) .Hydroxylation of same position when X= NH is performed slowly although when X=S, there is no reaction **[59]**.

Bioconversion of 3-acetoxypregna-5,16-diene-20-one (16-DPA) to androsta-1,4-diene-3,17-dione was performed by using mixed bacterial culture containing *Pseudomonas diminuta* and *Comamonas acidovorons*. Using either sequential or co-cultivation gave the same results. Although single culture was tried, there was no conversion obtained **[60]**.

In the study of Yazdi *et al.* it is stated that nandrolone decanoate was transformed into products, estr-4-en-3,17-dione, 17β-hydroxyestr-4-en-3-one, 15α-hydroxyestr-4-en-3,17-dione and 15α,17β-dihydroxyestr-4-en-3-one with ester hydrolysis, oxidation and hydroxylation respectively by *Acetomonium strictum* **[61]**.

Aspergillus terreus was used in the transformation of androstenedione into testosterone and testololactone by means of 17-carbonyl reduction and Baeyer-Villiger oxidation respectively **[62]**.



[4]

(19) **(20)**

Figure 20. Reaction scheme of de-acetylation of 3β,17β-diacetoxy-5α-androstane

Regioselective deacylation reaction of fully acetylated steroids is also performed by enzyme, e.g. *Candida rugosa* lipase-catalyzed transesterification of 3β,17β-diacetoxy-5α-androstane **(19)** to 17β-acetoxy-5α-androstan-3β-ol **(20)** with a successful yield 68 % **[4]**.

1.3. Fungi

The fungi (mycophyta) are eukaryotes which may be derived from the colorless representatives of the unicellular algae. Since they are not capable of forming plastids, they are heterotrophic. They live as saprophytes or parasites in fresh water (rarely in salt water) and on land. The saprophytes, and also many of the parasites, can be cultivated in the laboratory **[32]**. The fungi embrace eukaryotic organisms variously referred to as molds, mildews, rusts, smuts, yeasts, mushrooms, and puffballs. Of the soil organism, the fungi as a group are the organotrophs primarily responsible for the decomposition of organic residues **[63]**.

Fungi are a unique group of organisms, different from all others in their behavior and cellular organization. They also have enormous range of activities – as pathogens of crop plants or humans, as decomposer organisms, as experimental "model organisms" for investigating genetics and cell biology, and as producers of many important metabolites. Thus, they have an enormous range of biochemical activities that are exploited commercially – notably the production of antibiotics (e.g. penicillins), **steroids** (for contraceptives), ciclosporins (used as immunosuppressants in transplant surgery), and enzymes for many purposes such as food processing, bioconversions in organic chemistry **[20]**.

1.3.1. *Fusarium* species

Fusarium is a large genus of filamentous fungi widely distributed in soil and in association with plants. Most species are harmless saprobes and are relatively abundant members of the soil microbial community **[65]** and they are active in the decomposition of cellulosic plant materials. *Fusarium* is one of the three major fungal genera producing toxins **[64]**. The main toxins produced by these *Fusarium* species are fumonisins and trichothecenes **[65]**.

Fusarium is a type fungus which exists in a group of Fungi imperfecti, class of Deuteromyces with the features of septate hyphae reproduced only by asexual conida, if sexual stage found, usually ascomycete **[63]**. The character which defines

the genus *Fusarium* is the production of septate, fusiform to crescent shaped conidia, termed macroconidia, with a foot shape basal cell and a more or less beaked apical cell **[64]**.

1.4. Aim of the work

In the first part of the study, the main aim was to test the ability of *Fusarium* species including *Fusarium roseum* OUT 4019, *Fusarium anguioides* OUT 4017, *Fusarium solani* OUT 4021, *Fusarium bulbigenum* OUT 4115 by reduction of ketone at C-3 and C-21 in addition to other positions and also by $^{1,2}\Delta$-dehydrogenation (Figure 21) on A and D rings, which are important regions containing many active regions having pharmaceutical importance.

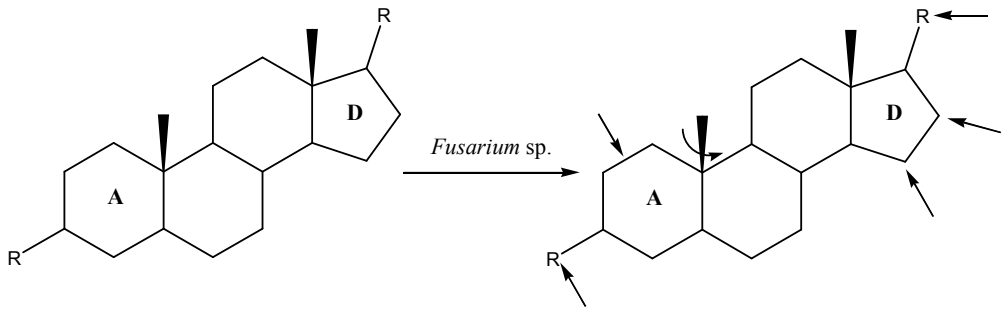


Figure 21. Scheme of purposed bioconversions of steroids by *Fusarium* species

Second part of the study covers the cleavage of aromatic methyl ethers using biotechnological techniques (Figure 22).

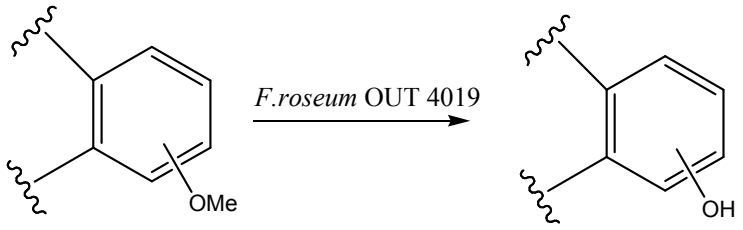


Figure 22. Scheme of purposed bioconversion of aromatic methyl ethers by *Fusarium roseum* OUT 4019.

CHAPTER 2

RESULTS AND DISCUSSION

2.1. STEROID BIOCONVERSIONS

2.1.1. Perspective of the work

Naturally occurring compounds are in a great interest since the early times of life of human beings. Natural products are important compounds which are synthesized by living organisms. They are divided into two groups according to their types: primary and secondary metabolites.

Primary metabolites are vital for the living organisms. These metabolites start to produce during growth phase and production continues throughout the life of the organisms. Thus, these compounds are widespread among all organisms.

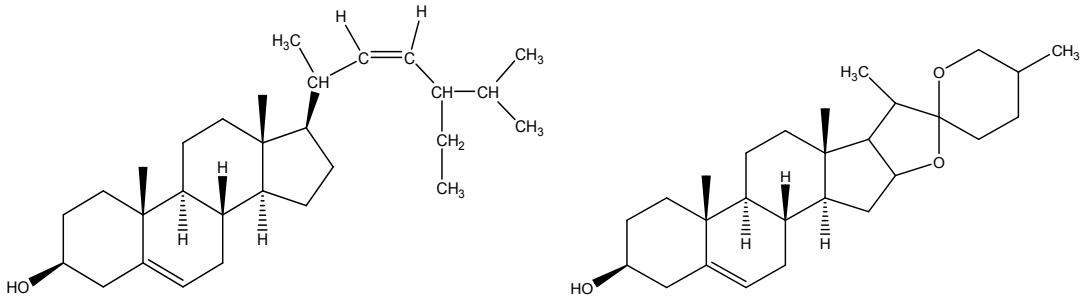
Hormones are one of the most important primary metabolites. They exist nearly in all multi cellular organisms. They are powerful signal molecules that have roles as regulators in the function of metabolism. In mammalians, they are involved in many functions such as regulation of blood sugar level, sodium-potassium level, degradation of fats and proteins, increasing muscle and bone synthesis, inhibition of inflammatory response etc. Steroids are the most important types of hormones providing sex hormones and adrenal cortex hormones, e.g. progesterone, and cortisone which have extensive application in birth control pills and anti-inflammation respectively.

On the other hand, conversion of steroids by using biotechnological methods is a highly growing area and promising an alternative method of steroid synthesis.

Biotransformation process has many advantages over conventional chemistry. It has environmentally safe and friendly conditions because its mild conditions and reaction media containing only water as a solvent instead of organic substance. The most important one is the specificity of enzymes besides their environmentally safe conditions. By stereo- and regio-specific properties of enzymes or microorganisms having those enzymes, desired selectivity on the product has been achieved easily. In drug development, producing products being highly pure and also having high enantiomeric purity and regio-specificity is important since each enantiomers of the molecule existing in drugs show different effects on the body. Producing a drug with a single enantiomer is possible with bioconversion methods. Therefore, in pharmaceuticals, this specificity gives the biotechnology the leading position to produce target specific drugs.

In bioconversion reactions, steroids are generally mostly studied substance in the pharmaceutical. There are many precursors which are used to synthesize the desired type of steroids. Most of them are isolated from the plant sources such as stigmasterol and diosgenin.

Stigmasterol **(21)** and Diosgenin **(22)** are plant steroids used as the starting material for a commercial synthesis of cortisone and sex hormones **[22]**.



(21) **(22)**

Figure 23. Starting materials for the synthesis of steroid hormones

Typical bioconversion reactions performed on steroidal substance are hydroxylation and oxidation reactions. In the literature there are many bioconversion reactions related with steroids. However, their yields are not high enough. Moreover, most of

the reactions give more than one products with the same type conversion at different positions. Therefore, the selectivities are also very low.

In this study, it was tried to improve the bioconversion methods of synthesizing steroids for obtaining high conversion and yield. To achieve the purpose, fungi as whole cells were used in the bioconversion of steroids, which were 4-androstene-3,17-dione and 4-cholesten-3-one, as substrates. Since these steroid structures have similarities with the important hormones such as cholesterol, estrogen, testosterone, progesterone, and aldosterone, conversion of the starting material into those is an easy and simple way to carry out the reaction by using microorganisms. Also, these steroid types have enone systems that have double bonds on the A ring (-ene) and ketones (-one) at C-3 position on A ring and also C-17 position on D-ring which have an important roles in the bioconversion process since these regions are chemically active regions and they can be easily converted into positions having chiral properties, important in the pharmaceutical industry.

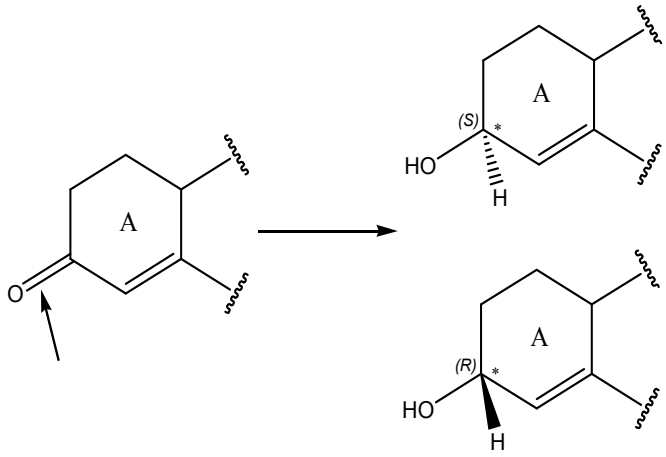


Figure 24. Reaction scheme for steroids to obtain a chiral center on A-ring

As indicated in Figure 24, ketone on A-ring can be reduced which could give two different enantiomeric products, only (R) or (S) or mixing of these two, generally. Chemically these products can also be obtained but selectivity is low so mostly racemic products (50 % ee) or near to that value is obtained. However, in

bioconversion with microorganisms, due to the selectivity of the enzyme, producing enantiomerically pure compounds is possible.

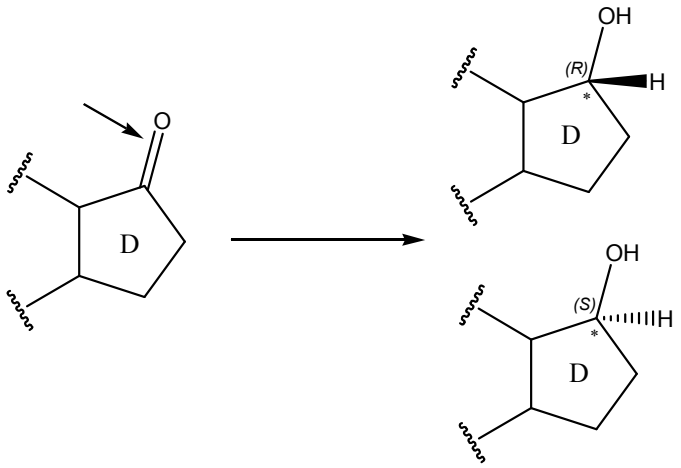


Figure 25. Reaction scheme for steroids to obtain a chiral center on D-ring

Similar reaction pathway is available for the reduction of the ketone on D-ring that can form two different enantiomeric products (Figure 25).

2.1.2. Screening of fungi

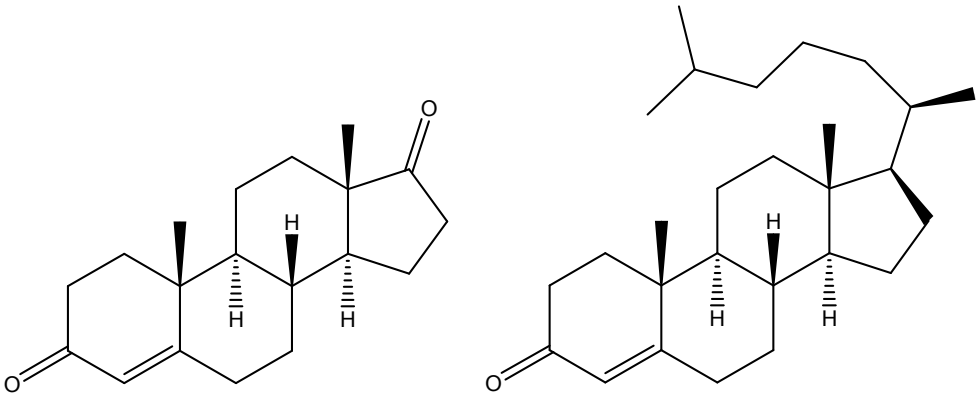
As a growth media, GP, PS, MGYP broths which are stated as suitable media for the growth of *Fusarium* species were used **[67].** In addition, GPA, PS agar, MGYP agar were used in order to obtain the spores of *Fusarium* species on agars for inoculation purposes.

In order to find the ability of selected microorganisms to transform steroids, *Fusarium* species including *Fusarium roseum* OUT 4019, *Fusarium solani* OUT 4021, *and Fusarium anguioides* OUT 4017 , *Fusarium bulbigenum* OUT 4115 were selected. These three species preferred due to their kinship, high efficiencies and also there was a preliminary work about *Fusarium roseum* bioconversion.

First of all, from the agars of stock cultures where the *Fusarium* species had been grown, inoculation was performed into broths by sterile loop. Microorganisms, especially fungi are widely distributed in the nature where they were isolated. Since they have specific growth regions containing different nutritional diversity as carbon, nitrogen, besides mineral and oxygen sources, the adaptation of those native microorganisms is a difficult procedure. Therefore, using a synthetic media, to obtain desired growth rates and also desired biomass values require additional studies. Because each of different media preparations contains different carbon, nitrogen and also mineral sources, microorganisms show different response to those parameters. According to those, since they grow in different style and different amounts in the media, these result in changing in conversion, yield and even in selectivity of the reaction that affect the overall bioconversion process directly.

For all *Fusarium* species including *Fusarium roseum* OUT 4019, *Fusarium solani* OUT 4021, *and Fusarium anguioides* OUT 4017, *Fusarium bulbigenum* OUT 4115 selected for this study, there was a growth observed in these broths. Obtained results were used in the biotransformation reactions. To achieve a sufficient biomass results, microorganisms were incubated at 25 °C for 4.5 days in shaker.

For bioconversion reactions, 4-androstene-3,17-dione **(18)** and 4-cholesten-3-one **(23)** which have enone systems were chosen as model compounds.



(18) **(23)**

Figure 26. Molecular structure of 4-androstene-3,17-dione and 4-cholesten-3-one

After the incubation, steroids, 4-androstene-3,17-dione **(18)** and 4-cholesten-3-one **(23)**, were added into the media with obtained biomass results by dissolving them in an organic solvent dimethylsulfoxide, DMSO, which is miscible in water easily. During the bioconversion period, reactions of *Fusarium* species were monitored by using thin layer chromatography, TLC, with a phosphomolybdic acid dye, PMA under $UV_{254\ nm}$ light for both substrate and products which are UV active.

In the first control experiment, 4-androstene-3,17-dione was used as substrate and it was shown that all *Fusarium* species converts substrate into any products. These steroid types which were used as starting materials for the bioconversion reactions by *Fusarium* species were used readily without performing any chemical synthesis because of their commercial availability.

Fusarium roseum OUT 4019 catalyzed the steroid bioconversion reaction in GP and MGYP broths. Whereas *Fusarium anguioides* OUT 4017 and *Fusarium solani* OUT 4015 catalyzed the reaction in PS and MGYP broths, *Fusarium bulbigenum* OUT 4115 catalyzed the reaction in only MGYP broth.

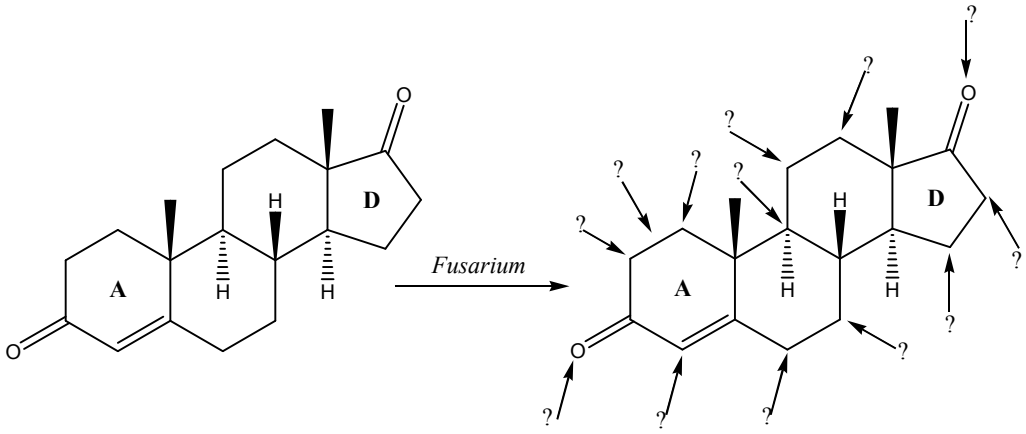


Figure 27. Possible sites for the **s**teroid bioconversion by *Fusarium* species

The conversion was kept and monitored with thin layer chromatography (TLC). When Rf values of the products were analyzed on TLC, it was easily seen that each species had catalyzed the steroid bioconversion reactions differently by producing

different products. However, it was not possible to determine the transformation type or product type by looking at only TLC data. Therefore, after work up, obtained crude products were purified by column chromatography and then analyzed by ^{1}H-NMR and ^{13}C-NMR spectrum and also GC-MS.

According to NMR and GC-MS spectrum data besides isolated yields, *Fusarium roseum* OUT 4019 had a best conversion in GP broth. While *Fusarium anguioides* OUT 4017 showed the highest performance in PS broth, *Fusarium solani* OUT 4021 did in MGYP broth. However, for *Fusarium bulbigenum* OUT 4115, although conversion was observed with 4-androstene-3,17-dione, any identification couldn't be performed. Therefore, after those experiments, by optimization studies it was tried to find the best condition to carry out this reaction in steroid conversion for obtaining high chemical conversion and yields.

The same procedure was performed for 4-cholesten-3-one as in the case of 4-androstene-3,17-dione. For all *Fusarium* species used, no conversion was observed because of low solubility of the 4-cholesten-3-one. Therefore, 4-androstene-3,17-dione was selected as a model substrate for the optimization of bioconversion studies by *Fusarium* species.

Besides these optimization studies, blank experiments for both steroid substrates, 4-androstene-3,17-dione and 4-cholesten-3-one were carried out in order to find the effect of media on the steroids during bioconversion periods. Blank experiments were monitored by TLC as in the case of bioconversion studies during two weeks. For both substances, there was no change observed in the media selected. To sum up, the two starting materials were identified as stable materials in those media.

2.1.3. Optimization studies for fungal bioconversion

Some parameters glucose concentration, solvent, growth media either agar or broth, inoculum size were changed to find out the optimum conditions with the hope having the best chemical yields in these reactions.

2.1.3.1. Effect of solvent

It is well known that fungus reactions proceed in aqueous media. Since the compounds used in this study are steroids, major problem is solubility in water, steroids only soluble to 0.1 - 1% in water. It is necessary to use organic solvents and emulsions for solution of substrates and products. However, although enzymes can be quite stable to organic solvents, membranes of cells are very sensitive and solvents cause disruption. This can create a problem when live cells are required with an active electron transport chain **[68]**. Therefore, it was necessary to find out a co-solvent;

-which could dissolve these substrates,
-give no harm to *Fusarium* species
-that is miscible with water so that the reaction will take place in a homogenous media.

For these purposes, three solvents: dimethysulfoxide (DMSO), ethanol and acetone were selected and tested to find out the suitable solvent which has the above mentioned properties.

These solvents were tested with substrates to see their solubilities. Three of them have an ability to dissolve 4-androstene-3,17-dione successfully.

Then, these solvents, 5 mL, were added separately into the sterilized media, in which *Fusarium* spp. were inoculated and incubated at 25 °C. Although all three have good dissolving abilities with a decreasing order: acetone, DMSO, and ethanol, ethanol and acetone were eliminated due to their ease to vaporize, even in the mixture with water. Therefore, during the reaction period, since conversion period and temperature (25 °C) was almost suitable for vaporizing totally form the media, these solvents did not solve the solubility problem to obtain the highest yield.

The results show that of three solvent, dimethylsulfoxide was the best one which provide the optimum conditions.

According to the studies performed by DMSO, high level of it shows toxic effect on some fungi types. Therefore, in order to decrease the level of toxicity, as a second co-solvent ethanol and acetone were studied by decreasing the amount of DMSO. Each *Fusarium* spp. showed different effect against solvent mixture. While *F. roseum* and *F. anguioides* showed better results against DMSO-acetone mixture, for *F. solani* higher yield was obtained with DMSO-ethanol mixture. Ethanol had a positive effect on conversion by utilizing as carbon source so it increased the yield **[57, 58]**.

Besides the studies of 4-androstene-3,17-dione, 4-cholesten-3-one was also studied to solve the solubility problem in the media. In order to find the best solvent to eliminate the solubility problem three powerful solvent were used as in the case of 4-androstene-3,17-dione. However, although acetone and DMSO are powerful solvents, they could not dissolve 4-cholesten-3-one totally when using suitable amount of solvent. Although it was possible to dissolve it by using high amount of solvent, it couldn't be applied since those amounts would be toxic for microorganisms. Besides, with ethanol as a solvent, solubility problem were achieved with increasing solvent amount at the beginning. However, bioconversion period was long so, and ethanol is volatile liquid, this problem did not completely eliminated. There was a conversion observed with ethanol as a co-solvent. However, due to the inabilities for the solubility of 4-cholesten-3-one conversions preventing the complete conversion, the obtained yields for all *Fusarium* species were very low to detect the structure of product by NMR and GC-MS analysis. Therefore, 4-androstene-3,17-dione was selected as a model substrate for the optimization of bioconversion studies of *Fusarium* species.

2.1.3.2. Effect of glucose concentration

It is known that glucose is the primary carbon source of living things all around the world. In the growth media, glucose concentration affects the conversion of substrate also the chemical yield indirectly by means of growth and biomass formation. Since the growth and continuity of life depends on the nutrition, when there is no enough nutrition, mainly containing carbon sources, microorganisms start to degrade the

starting material using as a carbon source as also stated by Sripalakit *et al.* .This affects the total conversion and yield of the product in many different ways such as producing side products which are difficult to eliminate from the product extract and needs further purification steps decreasing the yield, and decrease the converted product because of using as also carbon source. To eliminate these problems, optimization must be required.

Glucose concentration study was performed only for *Fusarium roseum* OUT 4019. Therefore, it was optimized only for it. Between the values of 2.5, 3.0 and 3.5 % w/v of glucose concentration was studied for GP (glucose-Peptone) media and 3.0 % w/v glucose concentration gave the best conversion and yield.

2.1.3.3. Effect of type of growth media

For microorganisms, growth media indirectly affect the conversion and yield of the bioconversion. When there is a successful growth of microorganism in the media, results of the reaction will be better.

F. solani OUT 4021 grown in PD agar as in the case of the others and this had a low yield to produce spore on the surface of agar. Having fresh and better grown spores in stock culture is important for obtaining high cell biomass in liquid media. In order to increase the spore yield, two different agars were tested: PDA (Potato Dextrose Agar and MGYP agar). According to results, on MGYP agar, growth of spores gave better results. This result was also applied for the broth and the same success was obtained for growth on MGYP broth when it was compared to the previously used broth, PSB. Therefore, MGYP was selected as growth media as an agar and broth for *F. solani* OUT 4015. The other two species *F. roseum* OUT 4019 in GP agar and broth and also *F. Anguioides* OUT 4017 in PS agar and broth were grown and showed successful bioconversion results when compared to old cultured samples.

2.1.3.4. Effect of inoculum size

Initial spore suspension added into growth media has a high effect on the conversion and yields of the product. However, when there is a growth of microorganism in the broth, conversion of substrate into product will be more easy and fast. Spores of *Fusarium* species were added into broths as a loop number. After the incubation period, 4.5 days, was finished, all studied *Fusarium* species, *F. roseum* OUT 4019, *F. solani* OUT 4021, *F. anguioides* OUT 4017 in broths were filtered and then wet cell weights were measured. To measure the dry cell weights, cell samples placed in an oven and kept there for one day. The optimized wet and dry cell weights are given in the Table 1.

Table 1. Wet and dry cell weights of *Fusarium* species after incubation

Species	Wet cell weight (g)	Dry cell weight (g)
Fusarium roseum OUT 4019	10.18	1.055
Fusarium solani OUT 4021	10.16	0.897
Fusarium anguioides OUT 4017	3.74	0.261

These results shown in Table 1 were obtained by using 250 mL GP broth for *F. roseum*, MGYP broth for *F. solani* and PS broth for *F. anguioides* with 4.5 days incubation period at 25 °C and 160 rpm for wet cell. Dry cell weights were obtained by keeping them in an oven at 80 °C for 1 days.

2.1.4. *Fusarium* mediated conversion of 4-androstene-3,17-dione

Three different *Fusarium* species, *F. roseum* OUT 4019, *F. anguioides* OUT 4017, *F. solani* OUT 4021 were used for the bioconversion of 4-androstene-3,17-dione with the optimized conditions.

2.1.4.1. Bioconversion of 4-androstene-3,17-dione with *F. roseum* OUT 4019

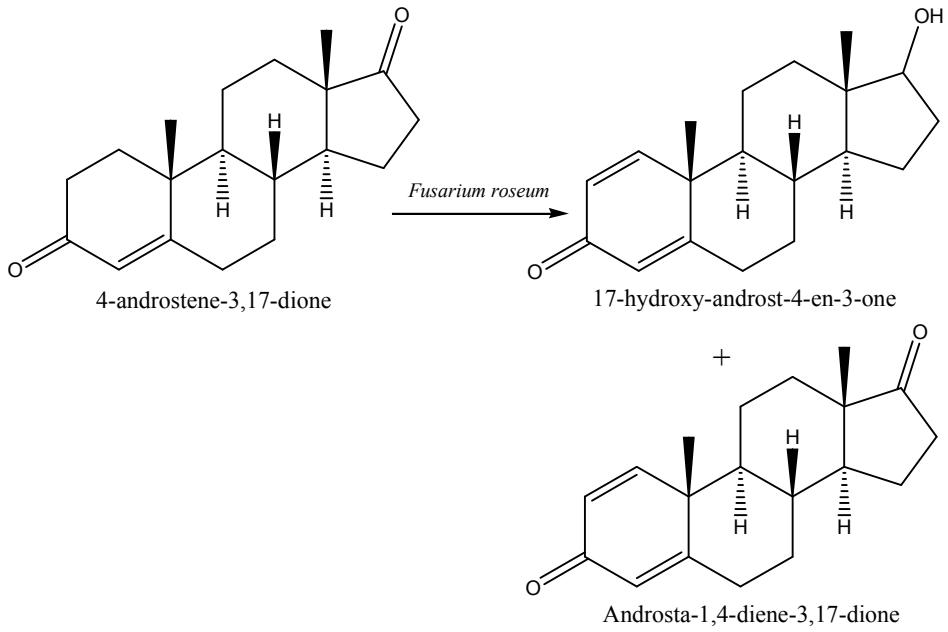


Figure 28. Reaction scheme of 4-androstene-3,17-dione by *Fusarium roseum*

The conversion reaction of 4-androstene-3,17-dione was carried out under following conditions:

- at 25 °C
- at 160 rpm
- 70 mg substrate
- 3 mL DMSO and 1 mL acetone as co-solvent

The bioconversion of 4-androstene-3,17-dione with *Fusarium roseum* OUT 4019 carried out under the optimized conditions described above. 500 mL flasks containing 250 mL growth media were inoculated from the stock culture. After 4.5 days of incubation period at 25 °C and at 160 rpm in incubator with shaker, 70 mg 4-androstene-3,17-dione was dissolved in 3 mL DMSO and 1 mL acetone and added into the growth media of *F. roseum* OUT 4019 and the reaction was monitored by TLC analysis that; within a day, bioconversion of 4-androstene-3,17-dione into two product 17-hydroxy-androsta-1,4-dien-3-one by $^{1,2}\Delta$-dehydrogenation and androsta-

1,4-diene-3,17-dione by reduction at C-17 ketone position in addition to $^{1,2}\Delta$-dehydrogenation could be seen. These products were identified by NMR and GC-MS spectra. In the results of ^{1}H-NMR spectra, when spectra are compared (two products and starting material), for both products, there are two doublet formations at 6.13, 6.13 and 6.93, 6.94 ppm chemical shifts respectively in addition to singlet 5.96, 5.98 ppm of olefinic proton on A-ring. Moreover, for 17-hydroxy-androsta-1,4-dien-3-one, at 3.55 ppm gives triplet due to the formation of ketone reduction on D-ring.

Table 2. Rf values of starting material and product of *Fusarium roseum*

Substance	Rf value	MW (Da)
4-androstene-3,17-dione	0.375	286
Androsta-1,4-diene-3,17-dione	0.3	284
17-hydroxy-androsta-1,4-dien-3-one	0.2	286

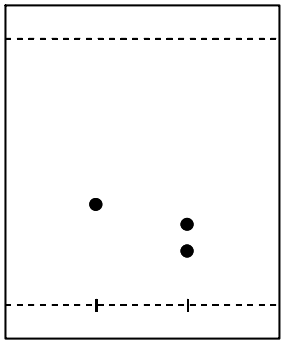


Figure 29. TLC profile of the starting material and products of *F. roseum*

When looked at the Rf values, as the molecular weight increases, those are decreased since higher MW weight products move less when compared to low ones. Even though one of the products having same MW with the starting material, their Rf values are different form each other. Therefore, besides MW, conformational or structural change in the molecule affect the movement of the molecule on TLC by changing Rf values.

The reaction was concluded 3.5 days after the addition of starting material. After work-up and purification of 17-hydroxy-androsta-1,4-dien-3-one, and androsta-1,4-diene-3,17-dione, they were obtained with 22 % and 54 % yields respectively.

Although the reaction was performed with complete conversion of substrate, yields of the products decreases due to the characteristics of products which have difficulty to isolate them separately from the crude product, losses during purification and separation, and maybe the most important factor that is the formation of two different products. However, by using advanced techniques, obtained yield could be increased much more.

2.1.4.2. Bioconversion of 4-androstene-3,17-dione with *F. anguioides* OUT 4017

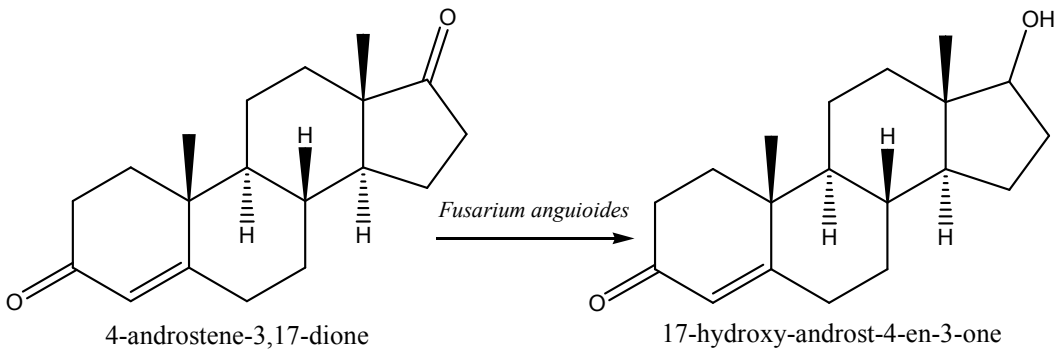


Figure 30. Reaction scheme of 4-androstene-3,17-dione by *Fusarium anguioides*

The conversion reaction of 4-androstene-3,17-dione was carried out under the same optimized conditions of *F. roseum* OUT 4019. After the addition of 70 mg 4-androstene-3,17-dione dissolved in 3 mL DMSO and 1 mL acetone into the growth media of *F. anguioides* OUT 4017, the reaction was monitored by TLC analysis that; bioconversion of 4-androstene-3,17-dione into 17-hydroxy-androst-4-en-3-one by reduction of ketone at C-17 position could be seen after approximately 7 days when the reaction proceeded. According to the NMR spectra, it can be identified that there is of triplet at 3.55 ppm, which does not exist in that of starting material.

Table 3. Rf values of starting material and product of *Fusarium anguioides*

Substance	Rf value	MW (Da)
4-androstene-3,17-dione	0.375	286
17-hydroxy-androst-4-en-3-one	0.325	288

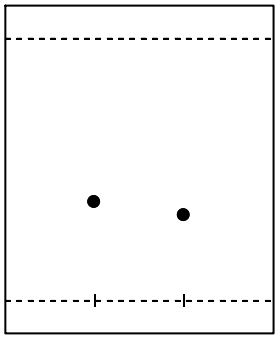


Figure 31. TLC profile of the starting material and product of *F. anguioides*

The reaction was concluded 21 days after the addition of starting material. After work-up and purification of 17-hydroxy-androsta-4-en-3-one, since it was not converted totally, 26 % yield, which is relatively low with respect to *F. roseum and F. solani*, was obtained. This result can be caused by less formation of biomass since biomass has an important effect on conversion and yield when looked at Table 1. If techniques increasing the biomass value of the *F. anguioides* can be applied, it is reasonable to increase the conversion and yield of the product as in the case of *F. roseum and F. solani.*

2.1.4.3. Bioconversion of 4-androstene-3,17-dione with *F. solani* OUT 4021

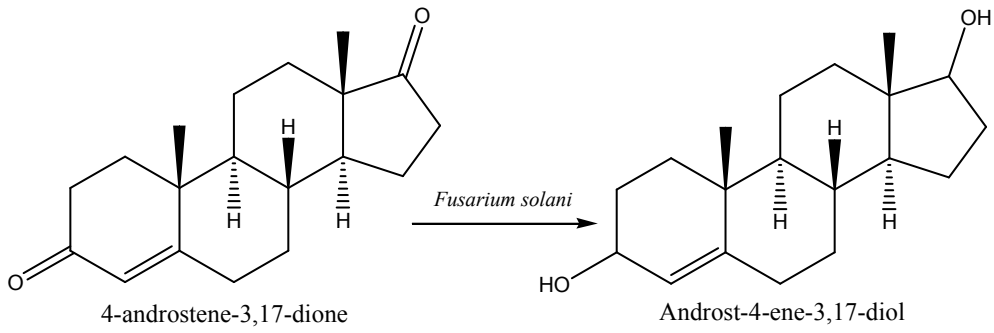


Figure 32. Reaction scheme of 4-androstene-3,17-dione by *Fusarium solani*

The conversion reaction of 4-androstene-3,17-dione was carried out under following conditions:

- at 25 °C
- at 160 rpm
- 70 mg substrate
- 5 mL DMSO and 10 mL ethanol as co-solvent

The bioconversion of 4-androstene-3,17-dione with *Fusarium solani* OUT 4021 carried out under the optimized conditions described above. 500 mL flasks containing 250 mL growth media were inoculated from the stock culture. After 4.5 days of incubation period at 25 °C and at 160 rpm in shaker incubator, 70 mg 4-androstene-3,17-dione was dissolved in 5 mL DMSO and 10 mL ethanol and added into the growth media of *F. solani* OUT 4021 and the reaction was monitored by TLC analysis that; within a day, bioconversion of 4-androstene-3,17-dione into androst-4-en-3,17-diol by reduction of ketones at both C-3 and C-17 positions could be seen. NMR spectra of the product prove that there are two reduction on A and D-ring ketones with triplet at 2.88 ppm and also quartet at 4.28 ppm.

Table 4. Rf values of starting material and product of *Fusarium solani*

Substance	Rf value	MW (Da)
4-androstene-3,17-dione	0.375	286
Androst-4-en-3,17-diol	0.125	290

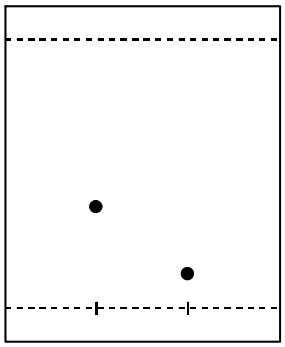


Figure 33. TLC profile of the starting material and product of *F. solani*

When the Rf values of products are compared, the lowest one is the product of *Fusarium solani*. This is obviously due to the molecular weight of the product besides molecular structure and conformational change of it.

The reaction was concluded 5 days after the addition of starting material. Once work-up and purification of androsta-4-en-3,17-diol, it was obtained with complete conversion and 90 % yield which is the highest among the *Fusarium* species.

2.2. DEMETHYLATION OF AROMATIC METHYL ETHERS

2.2.1 Demethylation of aromatic methyl ethers by fungi

Demethylation is widely used in organic chemistry. By using different regents such as boron reagents (boron trichloride, boron trifluoride in acetic anhydride, and diborane or sodium borohydride in the presence of iodine) **[69]** and also by chloroaluminate ionic liquid reagents, [TMAH][Al_2Cl_7] **[70]** these reactions were performed. However, the cleavage of methyl ethers from aromatic ring has not been observed yet by bioconversion process.

In this new study, cleavage of the methyl ether from aromatic ring was performed with microbiological source which is fungus.

Among the used *Fusarium* spp. in the steroid bioconversion studies, *Fusarium roseum* OUT 4019 achieved this bioconversion successfully.

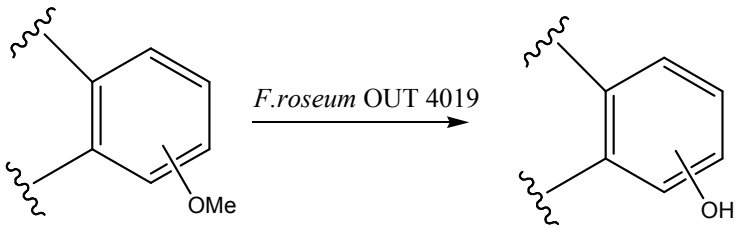


Figure 34. Scheme of proposed bioconversion of aromatic methyl ethers by *Fusarium roseum* OUT 4019

For this bioconversion study, six different starting materials were used: 4-methoxybenzoic acid, 6-methoxytetralone, 5-methoxytetralone, 2-methoxynaphthalene, 5-methoxyindanone, (E)-methyl-4-(4-methoxyphenyl)-2-oxobut-3-enoate which have aromatic ring and methyl ether substituent binding to the ring.

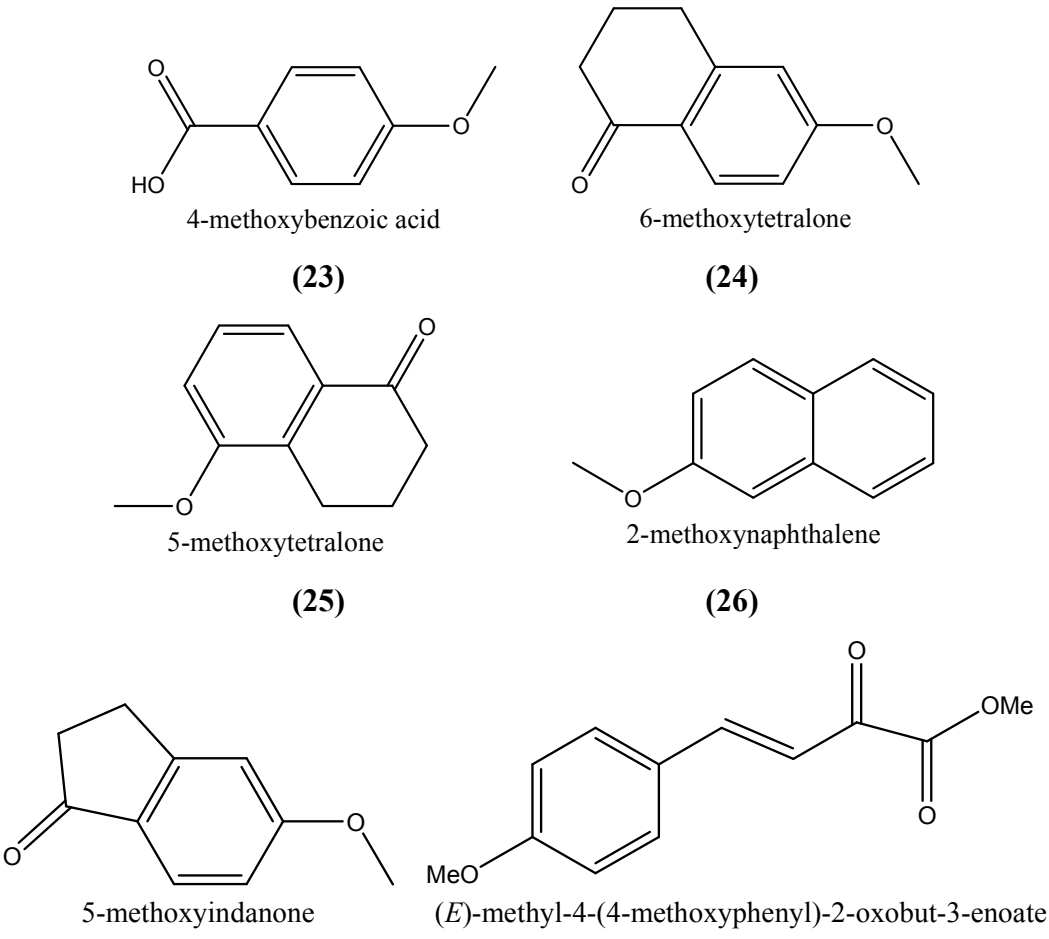


Figure 35. Methyl ether substituted compounds used in bioconversion

Reaction conditions optimized during the steroid conversion was also applied to 4-methoxybenzoic acid, 6-methoxytetralone, 5-methoxytetralone, 2-methoxynaphthalene, 5-methoxyindanone, (E)-methyl-4-(4-methoxyphenyl)-2-oxobut-3-enoate conversions as a reference.

70 mg of those substances were dissolved in 5 mL DMSO and added into the GP (Glucose-Peptone) media (400 mL) and incubated with *Fusarium roseum* OUT 4019. Reaction was monitored with TLC (1:1 EtOAc: Hexane for 6-methoxy, 5-methoxytetralone and 5-methoxyindanone, 1:3 EtOAc: Hexane for 2-methoxynaphthalene and 4-methoxybenzoic acid and (E)-methyl-4-(4-methoxyphenyl)-2-oxobut-3-enoate until no more change were observed after 14 days of incubation.

After stopping the reactions, whereas there is a product formation on 6-methoxytetralone, 5-methoxytetralone, (E)-methyl-4-(4-methoxyphenyl)-2-oxobut-3-enoate, there is no product observed for the bioconversion of 2-methoxynaphthalene, 5-methoxyindanone and 2-methoxynaphthalene.

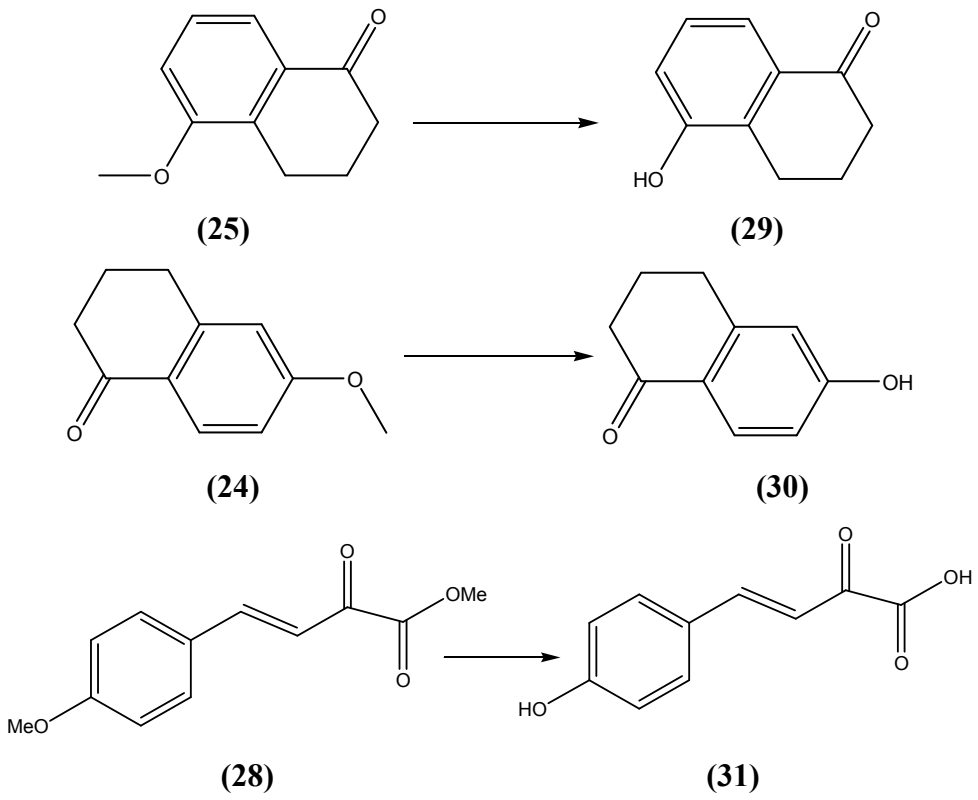


Figure 36. Demethylation reactions performed by *Fusarium roseum*

After work-up (involves filtration, extraction, and evaporation) of the crude product, purification was performed by flash column chromatography. However, as the yield of the products were too low during the bioconversion and the presence of unwanted side products made this technique impossible to be used. Presence of the products was proved by ^{1}H-NMR analysis. According to the starting material, around 3.5 ppm methoxy peaks were observed but, for the ^{1}H-NMR products obtained after work-up there was no methoxy peak. The bioconversion reactions of the four products are still under investigation.

CHAPTER 3

MATERIAL AND METHODS

3.1. Materials

Fungal bioconversion reactions were carried out in Edmund Bühler Johama Otto GMBH rotary shaker.

Flash column chromatographies were run on silica gel (Redi*sep* Flash column 4g (max. pressure 45PSI (3.1 bar)) with the solvent mixture indicated.

Thin layer chromatography was performed on commercial silica gel plates (Macherey-Nagel, 0.2 mm Silica Gel 60 with fluorescent indicator UV_{254}) that were developed by immersion in 5 % phosphomolybdic acid (PMA) in 95 % ethanol.

Preparative thin layer chromatography was performed on commercial silica gel plates (Macherey-Nagel, 2 mm silica gel, SIL G-200 UV_{254}, with UV_{254} indicator) was used with 1: 3 EtOAc - Hexane when the product was need to be purified further for NMR analysis.

All solvents were freshly distilled before use and stored.

^{1}H-NMR spectra were obtained on Bruker DPX400 instrument in $CDCI_3$ and CCI_4. Chemical shifts are expressed in ppm downfield from TMS; the ^{1}H-NMR data is present in the order: δ value of the signal, integrated number of protons, peak multiplicity (abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br., broad) and coupling constants in Hertz.

GC-MS spectra were determined using a ThermoQuest (TSP) TraceGC-2000 series equipped with phenomenex Zebron ZB-5 capillary column (5 % phenylmethylsiloxane, 30 m, 250 μm); T_{GC} (injector) = 250 °C, T_{MS} (ion source) = 200 °C, time programme (oven): $T_{0\ min.}$ = 60 °C, $T_{3\ min.}$ = 60 °C, $T_{14\ min.}$ = 280 °C (heating rate is 20 °C min^{-1}), $T_{20\ min.}$ = 280 °C, MS: ThermoQuest Finnigan multi mass (EI, 70 eV).

4-androstene-3,17-dione and 4-cholestene-3-one were purchased form ICN Biomedicals Inc. company.

All other reagents were analytical grade and were purchased form J. T. Baker and Lab Scan companies and also used without further purification.

3.2. Agar preparations for fungal growth

Fusarium roseum OUT 4019, *Fusarium solani* OUT 4021, *Fusarium anguioides* OUT 4017 species were inoculated into the agar for using in the succeeding bioconversion reactions performed in broth.

3.2.1. Agar preparations for *F. roseum* OUT 4019

For 100 mL agar (glucose - peptone agar, GPA):

3 % w/v Glucose (3.3 % glucose monohydrate)
1 % w/v Peptone
1.5 % w/v Agar bacteriological powder

Dilute to 100 mL with distilled water. Sterilize in autoclave for 15 minutes at 121 °C in a heat stable bottle. After cooling down to about 50 °C, pour liquid agar into Petri plates to solidify it in sterile conditions.

3.2.2. Agar preparations for *Fusarium solani* OUT 4021

For 100 mL agar (malt extract - glucose - yeast - peptone agar, MGYP agar):

0.3 % w/v Malt extract

1 % w/v Glucose (1.1 % glucose monohydrate)
0.3 % w/v Yeast extract
0.5 % w/v Peptone
1.5 % w/v Agar bacteriological powder

Dilute to 100 mL with distilled water. Sterilize in autoclave for 15 minutes at 121 °C in a heat stable bottle. After cooling down to about 50 °C, pour liquid agar into Petri plates to solidify it in sterile conditions.

3.2.3. Agar preparations for *Fusarium anguioides* OUT 4017

For 100 mL agar (potato extract- sucrose agar, PSA):

4 % w/v Potato extract
2 % w/v Sucrose
1.5 % w/v Agar bacteriological powder

Dilute to 100 mL with distilled water. Sterilize in autoclave for 15 minutes at 121 °C in a heat stable bottle. After cooling down to about 50 °C, pour liquid agar into Petri plates to solidify it in sterile conditions.

3.3. Broth preparations for fungal growth

In this study, *Fusarium roseum* OUT 4019, *Fusarium solani* OUT 4021, *Fusarium anguioides* OUT 4017 species were inoculated into the broths to perform the bioconversion reactions of 4-androstane-3,17-dione.

3.3.1. Broth preparations for *F. roseum* OUT 4019

For 250 mL broth (glucose - peptone, GP):

3 % w/v Glucose (3.3 % glucose monohydrate)
1 % w/v Peptone

Dilute to 250 mL with distilled water. Sterilize in autoclave for 15 minutes at 121 °C.

3.3.2. Broth preparations for *Fusarium solani* OUT 4021

For 250 mL broth (malt extract - glucose - yeast - peptone broth, MGYP broth):

0.3 % w/v Malt extract

1.0 % w/v Glucose (1.1 % glucose monohydrate)

0.3 % w/v Yeast extract

0.5 % w/v Peptone **[67]**

Dilute to 250 mL with distilled water. Sterilize in autoclave for 15 minutes at 121 °C

.

3.3.3. Broth preparations for *Fusarium anguioides* OUT 4017

For 250 mL broth (potato extract- sucrose broth, PSB):

4 % w/v Potato extract

2 % w/v Sucrose **[67]**

Dilute to 250 mL with distilled water. Sterilize in autoclave for 15 minutes at 121 °C.

3.4. Fungus preparation for experiments

In this study, *Fusarium roseum* OUT 4019, *Fusarium solani* OUT 4021, *Fusarium anguioides* OUT 4017 species were used for bioconversion.

These three species were obtained from "Osaka University Culture Collection".

The preparation of *Fusarium roseum* OUT 4019 was maintained on the GP agar from stock culture described above. The streaked plates were incubated at 25 °C for 6-7 days for spore production and then stored at 4 °C until use. Since *Fusarium* is a filamentous fungus that do not produce colony during incubation, it is not possible to count the colony or cell number, these species inoculates as loop numbers. After their inoculation into the sterile flasks containing the media and they were grown in rotary shaker at 25 °C for 4.5 days. After 4.5 days, dissolving substrate was added into these media.

For the preparation of *Fusarium solani* OUT 4021, *Fusarium anguioides* OUT 4017, same procedure was applied. However, growth of spores of *F. solani* OUT 4021 in a dark place due to the high spore formation capacity in dark place.

3.5. Synthesis of 17-hydroxy-androsta-1,4-dien-3-one and androsta-1,4-diene-3, 17-dione from 4-androstene-3,17-dione with *F. roseum* OUT 4019

Method for synthesis of 17-hydroxy-androsta-1,4-dien-3-one and androsta-1,4-diene-3,17-dione:

70 mg of starting material 4-androstene-3,17-dione was dissolved in the solvent mixture of 3 mL DMSO and 1 mL acetone as co-solvent and was added to the media containing *F. roseum* OUT 4019 (grown at 25 °C for 4.5 days). The reaction was checked with TLC (1:1 EtOAc - Hexane).

350 mL of EtOAc was added into the media then the organic layer was separated and washed with saturated NaCI solution and dried over anhydrous $MgSO_4$. The crude products was separated and purified by flash column chromatography (1:6 EtOAc - Hexane).

17-hydroxy-androsta-1,4-dien-3-one was obtained in 22 % molar yield as light reddish colored crystals in 3.5 days. androsta-1,4-diene-3,17-dione was obtained in % 54 molar yield as deep reddish colored crystals in 3.5 days.

For 4-androstene-3,17-dione as a starting material:

^{1}H-NMR (400 MHz, $CDCI_3$-CCI_4): δ (ppm) 5.65 (s, 1 H), 0.85-2.43 (m, 25 H)

^{13}C-NMR (400 MHz, $CDCI_3$-CCI_4): δ (ppm) 218.75, 198.00, 169.06, 124.26, 76.99, 76.67, 53.89, 50.87, 47.29, 38.54, 35.75, 33.78, 32.47, 31.35, 30.79, 21.74, 20.31, 17.37, 13.64.

For 17-hydroxy-androsta-1,4-dien-3-one:

^{1}H-NMR (400 MHz, $CDCI_3$-CCI_4): δ (ppm) 0.74-2.46 (m, 20 H), 3.55 (t, *J = 8.4 Hz*, 1 H), 5.96 (s, 1H), 6.13 (d, *J= 10 Hz*, 1 H), 6.94 (d, *J = 10.4 Hz*, 1 H).

For androsta-1,4-diene-3,17-dione:

^{1}H-NMR (400 MHz, $CDCI_3$-CCI_4): δ (ppm) 0.82-2.48 (m, 21 H), 5.98 (s, 1 H), 6.14 (d, *J= 10* Hz, 1 H), 6.94 (d, *J= 10* Hz, 1 H).

3.6. Synthesis of 17-hydroxy-androst-4-en-3-one, from 4-androstene-3,17-dione with *F. anguioides* 4017

Method for synthesis 17-hydroxy-androst-4-en-3-one was the same as that described above. 17-hydroxy-androst-4-en-3-one was obtained in 26 % molar yield as light yellow colored crystals in 21 days.

^{1}H-NMR (400 MHz, $CDCI_3$-CCI_4): δ (ppm) 5.63 (s, 1H), 3.55 (t, J = 8.4, H), 0.72-2.37 (m, 26 H).

3.7. Synthesis of androst-4-ene-3,17-diol from 4-androstene-3,17-dione with *F. solani* 4021

Method for synthesis of androst-4-ene-3,17-diol:

70 mg of starting material 4-androstene-3,17-dione was dissolved in the organic mixture of 5 mL DMSO and 10 mL EtOH as a co-solvent and used as a secondary carbon source. Then, it was added to the media containing *F. solani* OUT 4021 (grown at 25 °C for 4.5 days). The reaction was checked with TLC (1:1 EtOAc - Hexane).

350 mL of EtOAc was added into the media then the organic layer was separated and washed with saturated NaCI solution and dried over anhydrous $MgSO_4$. The crude

product was separated and purified by flash column chromatography (1:6 EtOAc - Hexane).

Androst-4-ene-3,17-diol was obtained in 90 % molar yield as yellow colored crystals in 5 days.

^{1}H-NMR (400 MHz, $CDCI_3$-CCI_4): δ (ppm) 0.87-2.42 (m, 27 H), 2.88 (t, *J = 13.8 Hz*, 1H), 4.28 (q, *J = 16 Hz*, 1 H), 5.64 (s, 1H).

^{13}C-NMR (400 MHz, $CDCI_3$-CCI_4): δ (ppm) 215.75, 199.07, 170.64, 123.74, 77.03, 76.71, 69.93, 57.18, 53.81, 50.25, 46.15, 38.66, 35.67, 33.73, 32.70, 31.86, 20.23, 17.46, 15.20.

CHAPTER 4

CONCLUSION

Steroid structures are present in all hormones and they are very important regulators which are used to control the body functions. They are widely used in pharmacological industry. Recently, production of steroids as pharmaceuticals carried out by chemical methods. However, nowadays, production methods have been directed to biotechnological pathway since their biotechnological modification is an important way to obtain interesting derivatives.

In this study, a new bioconversion method is developed for the synthesis of many different steroid derivatives. By using this method, steroids with A-ring enone and D-ring keto functionality are converted into important pharmaceutical derivatives by *Fusarium* species including *Fusarium roseum* OUT 4019, *Fusarium solani* OUT 4021, *and Fusarium anguioides* OUT 4017 with high conversion and yields.

As a model system 4-androstene-3,17-dione and 4-cholesten-3-one are used and reaction of 4-androstene-3,17-dione with *F. roseum* converted into androsta-1,4-diene-3,17-dione by $^{1,2}\Delta$- dehydrogenation and 17-hydroxy-androsta-1,4-dien-3-one, by reduction of ketone at C-17 in addition to $^{1,2}\Delta$-dehydrogenation and with *F. anguioides* into 17-hydroxy-androst-4-en-3-one by reduction of ketone at C-17 and with *F. solani* into androst-4-ene-3,17-diol by reduction of ketones at both C-3 and C-17. These bioconversions were resulted in 22 %, 54 %, 26 %, 90 % yields, respectively. Besides, 4-cholesten-3-one was also tested with these microorganisms, however, there was no successful result obtained.

The reductions and dehydrogenations in the presence of *Fusarium* species, is a new, low cost, easily applicable method which is a good sample for green chemistry for synthesizing the important pharmaceutical compounds.

The results are summarized in Figure 37:

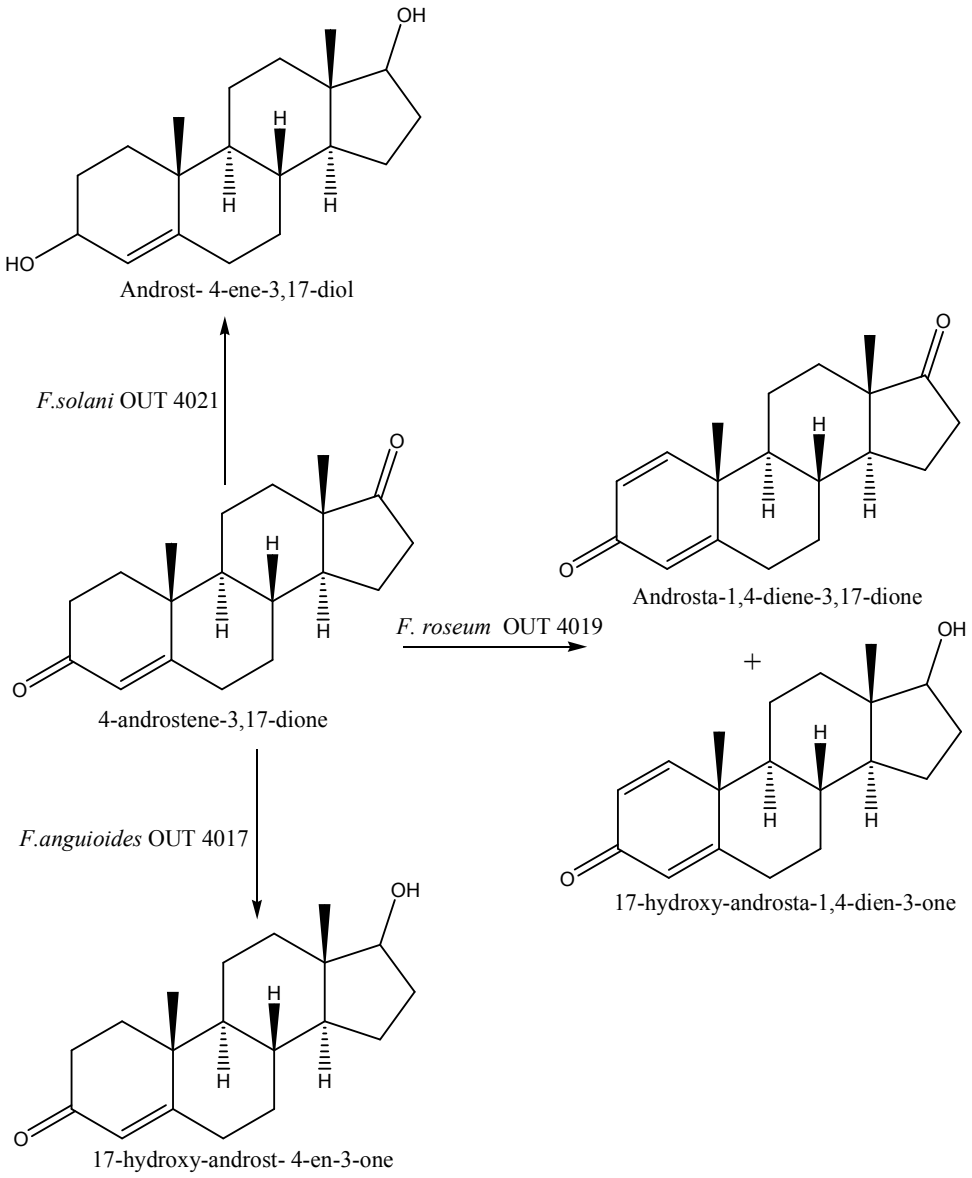


Figure 37. All steroid conversions performed by *Fusarium* species

In another study, demethylation of aromatic methyl ethers was studied by *Fusarium roseum* OUT 4019. For bioconversion of 6-methoxytetralone, 5-methoxytetralone and (E)-methyl,4-(4-methoxyphenyl)-2-oxobut-3-enoate, demethylated product formations were observed. However, 2-methoxynapthalene, 4-methoxybenzoic acid and 5-methoxyindanone demethylation reactions were not observed.

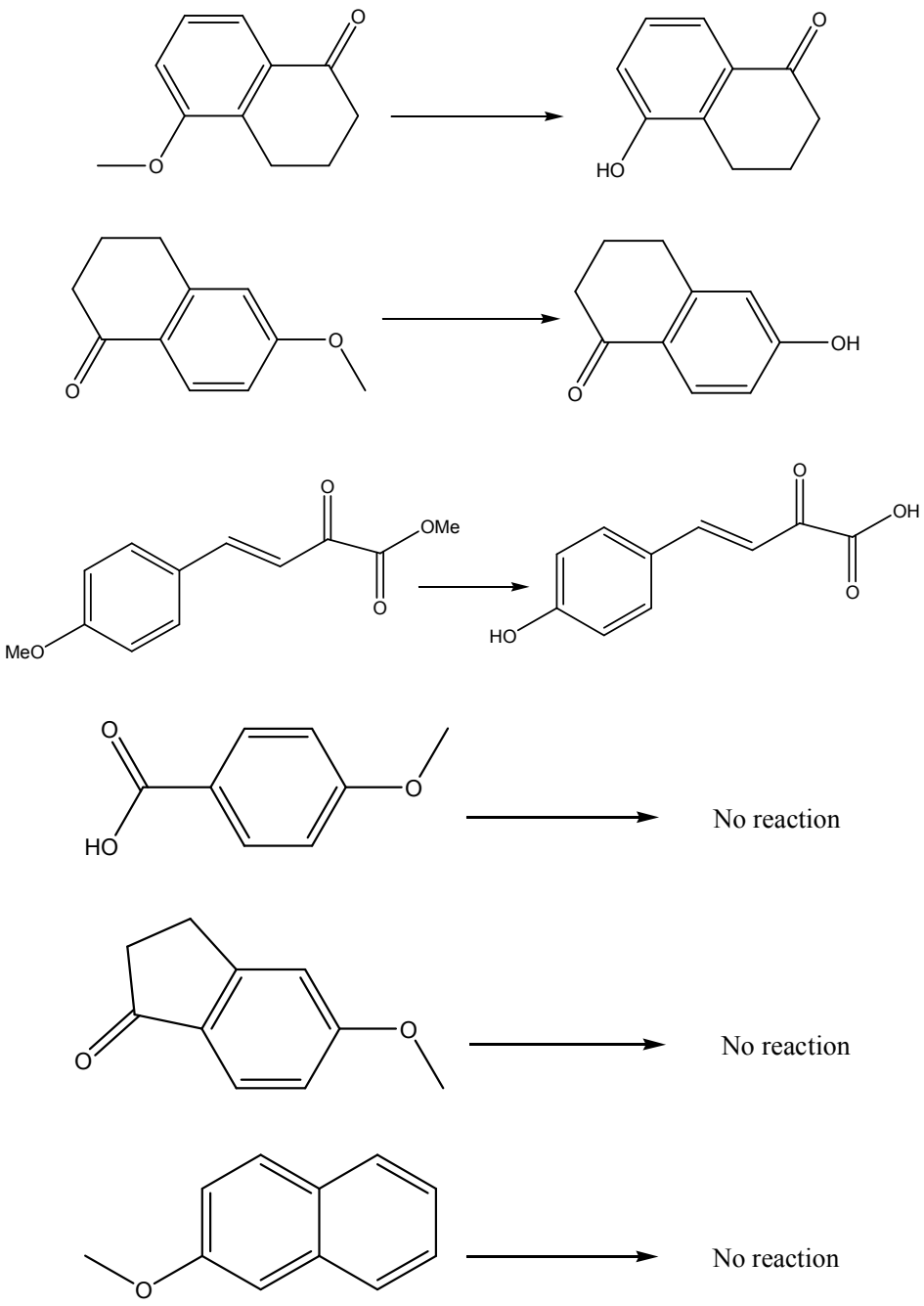


Figure 38. Bioconversion schemes of aromatic methyl ethers by *Fusarium roseum* OUT 4019

REFERENCES

[1] The World Wide Web; http://en.wikipedia.org/ last viewed 17.12.2007

[2] Kun, L. Y., *Microbial Biotechnology Principles and Applications*, second edition, World Scientific, Singapore, **2006**.

[3] Anke, T., *Fungal Biotechnology*, Chapman& Hall, London, **1997**.

[4] Koskinen, A. M. P., *Enzymatic Reactions in Organic Media*, Blackie Academic & Professional, Glasgow, **1996**.

[5] Bains, W., *Biotechnology form A to Z*, third edition, Oxford University Press, New York, **2004**.

[6] Mahato, S. B., Garai, S., *Steroids*, **1997**, 62, 332-345.

[7] Pajic, T., Vitas, M., Zigon, D., Pavko, A., Kelly, S. L., Komel, R., *Yeast,* **1999**, 15, 639–645.

[8] Swizdor, A., Kolek, T., *Steroids*, **2005**, 70, 817–824.

[9] Neslihanoğlu, R., Syntheses of Bioactive Compounds Using Biotechnological Methods: Fungal Culture Mediated Syntheses of Chiral α-hydroxy Ketones and Diols, Biotechnology Thesis, September-**2001**, METU.

[10] Mandal, D., Ahmadb, A., Khanb, M.I., Kumar, R., *Journal of Molecular Catalysis A: Chemical*, **2002**, 181, 237–241.

[11] Verdin, A., Saharaqui, A. L., Laruelle, F., Grandmougin-Ferjani, A., Durand, R., *Mycological research*, **2006**, 110, 4, 479 – 484.

[12] Daiber, A., Shoun, H., Ullrich, V., *Journal of Inorganic Biochemistry*, **2005**, 99, 185–193.

[13] Saunders, R. P., Hardman, R., *Biotechnology and Bioengineering*, **1985**, 27, 825-831.

[14] Swizdor, A., Szpineter, A., Kolek, T., *Journal of Molecular Catalysis B: Enzymatic*, **2006**, 42, 10–13

[15] Cai, W., Li, J., Zhang, Z., *Journal of Hazardous Materials*, **2007**, 148, 38–42.

[16] Sua, J., Xua, J., Lu, W., Lin, G., *Journal of Molecular Catalysis B: Enzymatic*, **2006**, 38, 113–118.

[17] Swizdor, A., Kolek, T., *Journal of Steroid Biochemistry and Molecular Biology*, **1998**, 67, No. 1, 63-69.

[18] The World Wide Web; http://www.chem.qmul.ac.uk/ last viewed 21.12.2007

[19] Briggs, M. H., Brotherton, J., *Steroid Biochemistry and Pharmacology*, Academic Press, New York, **1970**.

[20] Deacon, J., *Fungal biology, fourth edition*, Blackwell Publishing, **2006**.

[21] Simmonds, R. J., *Chemistry of Biomolecules: An Introduction*, The Royal Society of Chemistry, Cambridge, **1992**.

[22] Solomons, T. W. G., *Organic Chemistry*, fifth edition, Wiley & Sons, Inc., Toronto, **1992**.

[23] Donova, M. V., *Applied Biochemistry and Microbiology,* **2007**, 43, No. 1, 1–14.

[24] Berg, J. M., Tymoczko, J. L., Stryer, L., Clarke, N. D., *Biochemistry, fifth edition*, W. H. Freman and Company, New York, **2002.**

[25] Voishvillo, N. E., Andryushina, V. A., Savinova, T. S., Stytsenko, T. S., Vasileva, N. A., Turova, T. P., Kolganova, T. V., Skryabin,K. G., *Applied Biochemistry and Microbiology,* **2003**, 39, No. 2, 152-157.

[26] Koskinen, A., *Asymmetric Synthesis of Natural Products*, John Wiley & Sons, Chichester , **1993**.

[27] Kreuzer, H., Massey, A., *Biology and Biotechnology, Science, Applications and Issues*, ASM Press, Washington D.C., **2005**.

[28] Vollhardt K. P. C., Schore, N. E., *Organic Chemistry, Structure and Function*, fifth edition, W. H. Freeman and Company, New York, **2007**.

[29] Numazawa, M., Handa, W., Matsuzaki, H., *Journal of Steroid Biochemistry & Molecular Biology*, **2006**, 101, 239–245.

[30] The World Wide Web; http://web.indstate.edu/ last viewed 25.12.2007

[31] Fokina, V. V., Arinbasarova, A. Y., Zubov, L. A., Lozinsky, V.L., Koshcheenko, A.K., *Applied Biochemistry and Microbiology*, **1995**, 31(2), 184-189.

[32] Prave, P., *Fundamentals of Biotechnology*, **1987**, VCH, New York.

[33] Numazawa, M., Handa W., *Chem. Pharm. Bull*., **2006**, 54, 4, 554-556.

[34] Moses, V., Cape, R.E., *Biotechnology, The Science and The Business*, Harwood Academic Publishers, New York, **1991**.

[35] Fernandes, P., Cruz, A., Angelova, B., Pinheiro, H. M., Cabral, J. M. S., *Enzyme and Microbial Technology*, **2003**, 32, 688–705.

[36] He, X., Liu, B., Wang, G., Wang, X., Su, L., Qu, G., Yao, X., *Journal of Steroid Biochemistry and Molecular Biology*, **2006**, 100, 87–94.

[37] Dorfman, R.I., Ungar, F., *Metabolism of Steroid Hormones*, Academic Press, New York, **1965**.

[38] Manosroi, J., Manosroi, A., Saraphanchotiwittaya, A., Abe, M., *Journal of Chemical Technology and Biotechnology*, **1999**, 74, 364-370.

[39] Glazer, A. N., Nikaido, H., *Microbial Biotechnology Fundamentals of Applied Microbiology*, W.H. Freeman and Company, **1998**.

[40] Moore-Landecker, E., *Fundamentals of the Fungi, fourth edition*, Prentice Hall, **1996**.

[41] Romano, A., Romano, D., Ragg, E., Costantino, F., Lenna, R., Gandolfi, R., Molinari, F., *Steroids*, **2006**, 71, 429–434.

[42] Adham, N. Z., El-Hady, A. A., Naim, N., *Process Biochemistry*, **2003**, 38, 897-902.

[43] Holland, H. L., Lakshmaiah, G., *Journal of Molecular Catalysis B: Enzymatic*, **1999**, 6, 83–88.

[44] Hanson, R. J., Hunter, A. C., *Phytochemistry*, **1998**, 49, No.8, 2359-2362.

[45] Veizina, C., Sehgal, S. N., Singh, K., *General Microbiology,* **1962,** Ayerst Research Laboratories, Canada.

[46] Sedlaczek, L., Dlugonski, J., Jaworski, A., *Applied Microbiology and Biotechnology*, **1984**, 20, 166-169.

[47] Somal, P., Chopra, C.L., *Applied Microbiol. and Biotech.***, 1985**, 21, 5, 267-269.

[48] Cotillon, A., Morfin, R., *Journal of Steroid Biochemistry and Molecular Biology*, **1999**, 68, 229-237.

[49] Huszcza E., Dmochowska-Gladysz, J., *Phytochemistry*, **2003**, 62, 155–158.

[50] Huszcza, E., Dmochowska-Gladysz, J., *Journal of Basic Microbiology*, **2003**, 43, 2, 113–120.

[51] Bartmanska, A., Dmochowska-Gladysz, J., *Enzyme and Microbial Technology*, **2007**, 40, 1615–1621.

[52] Songtao, B., lianxiang, D., Liming, Z., Fuping, L., *Process Biochemistry*, **2005**, 40, 3309–3313.

[53] Smolders, A.J.J., Pinheiro, H. M., Noronha, P., Cabral, J. M. S., *Biotechnology and Bioengineering*, **1991**, 38, 1210-1217.

[54] Constantinides, A., *Biotechnology and Bioengineering*, **1980**, 22, 119-136.

[55] Manosroi, J., Sripalakit, P., Manosroi, A., *Journal of Chemical Technology and Biotechnology*, **1998**, 73, 203-210.

[56] Boeren, S., Laane, C., *Biotechnology and Bioengineering*, **1987**, 29, 305-309.

[57] Llanes, N., Pendas, J., Falero, A., Perez, C., Hung, B. R., Moreira, T., *Journal of Steroid Biochemistry & Molecular Biology*, **2002**, 80, 131–133.

[58] Sripalakit, P., Wichai, U., Saraphanchotiwitthaya, A.**,** *Journal of Molecular Catalysis B: Enzymatic*, **2006**, 41, 49–54.

[59] Faber, K., *Biotransformations in Organic Chemistry*, A textbook, 4th edition, Springer-Verlag, Berlin, **2000**.

[60] Patil, S., Shukla, A., Shinde, K., Banerjee, T., *Letters in Applied Microbiology*, **2002**, 35, 95–97

[61] Yazdi, M. T., Zanjanian, S. M., Faramarzi, M. A., Amini, M., Amani, A., Abdi, K., *Arch. Pharm. Chem. Life Sci.*, **2006**, 339, 473 – 476.

[62] Faramarzi,M. A., Yazdi, M. T., Amini, M., Mohseni, F. A., Zarrini, G., Amani, A., Shafiee, A., *World Journal of Microbiology and Biotechnology*, **2004**, 20, 657–660.

[63] Paul, E.A., Clark, F.E., *Soil Microbiology and Biochemistry*, Academic Press, Inc., California, **1989**.

[64] Pitt, J.I., Hocking, A.D., *Fungi and Food Spoilage*, second edition, Blackie Academic & Professional, Cambridge, **1997.**

[65] Howard, D. H., *Pathogenic Fungi in Humans and Animals, 2nd edition*, Marcel Dekker, **2003**.

[66] Devlin, M.T., *Textbook of Biochemistry with Clinical Correlations*, sixth edition, Wiley-Liss., New York, **2006**.

[67] Atlas, R.M., *Handbook of microbial media*, CRC press, Boca Raton, Fla., **2004.**

[68] The World Wide Web; http://www-biol.paisley.ac.uk/ last viewed 25.12.2007

[69] Jung, M.E, Lyster, M. A., *Organic synthesis*, Coll., **1988**, 6, 353; 1979, 59, 35

[70] Kemperman, G.J., Roeters, T.A., and Hilberink, P.W., *European Journal of Organic Chemistry*, **2003**, 1681-1686

APPENDIX A

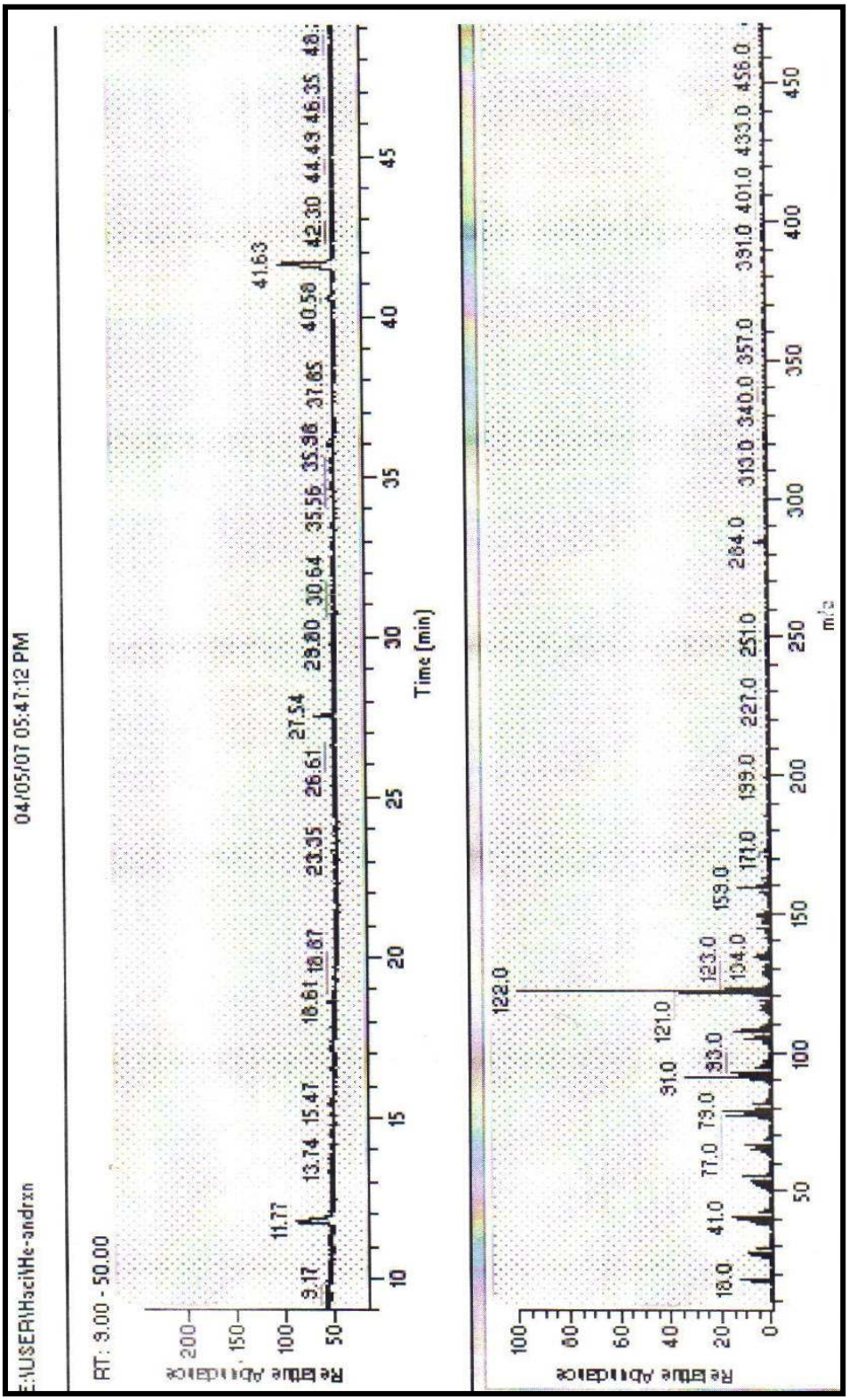


Figure 39. GC-MS spectra of androsta-1,4-diene-3,17-dione

APPENDIX B

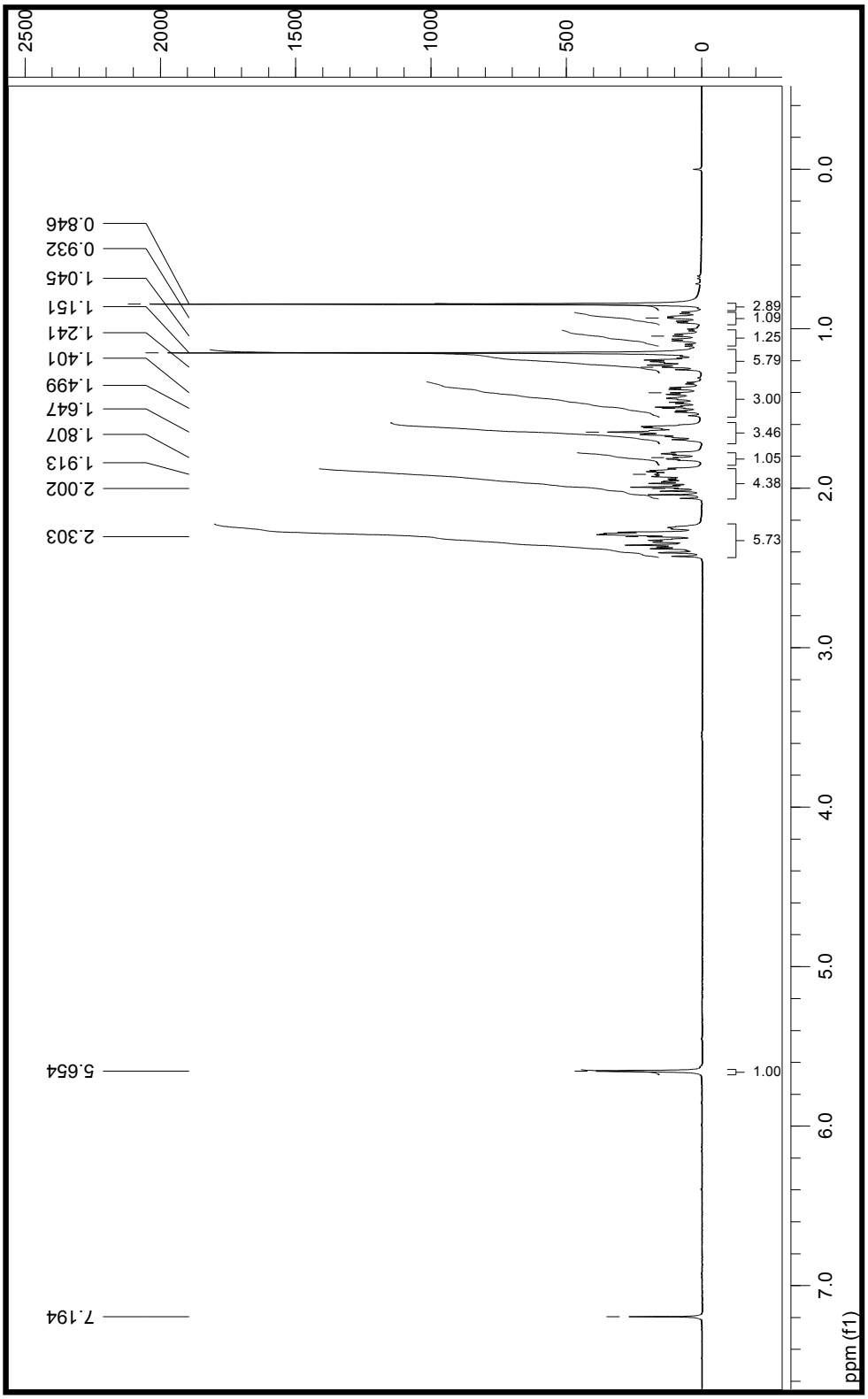


Figure 40. ^{1}H NMR spectrum of starting material, 4-androstene-3,17-dione

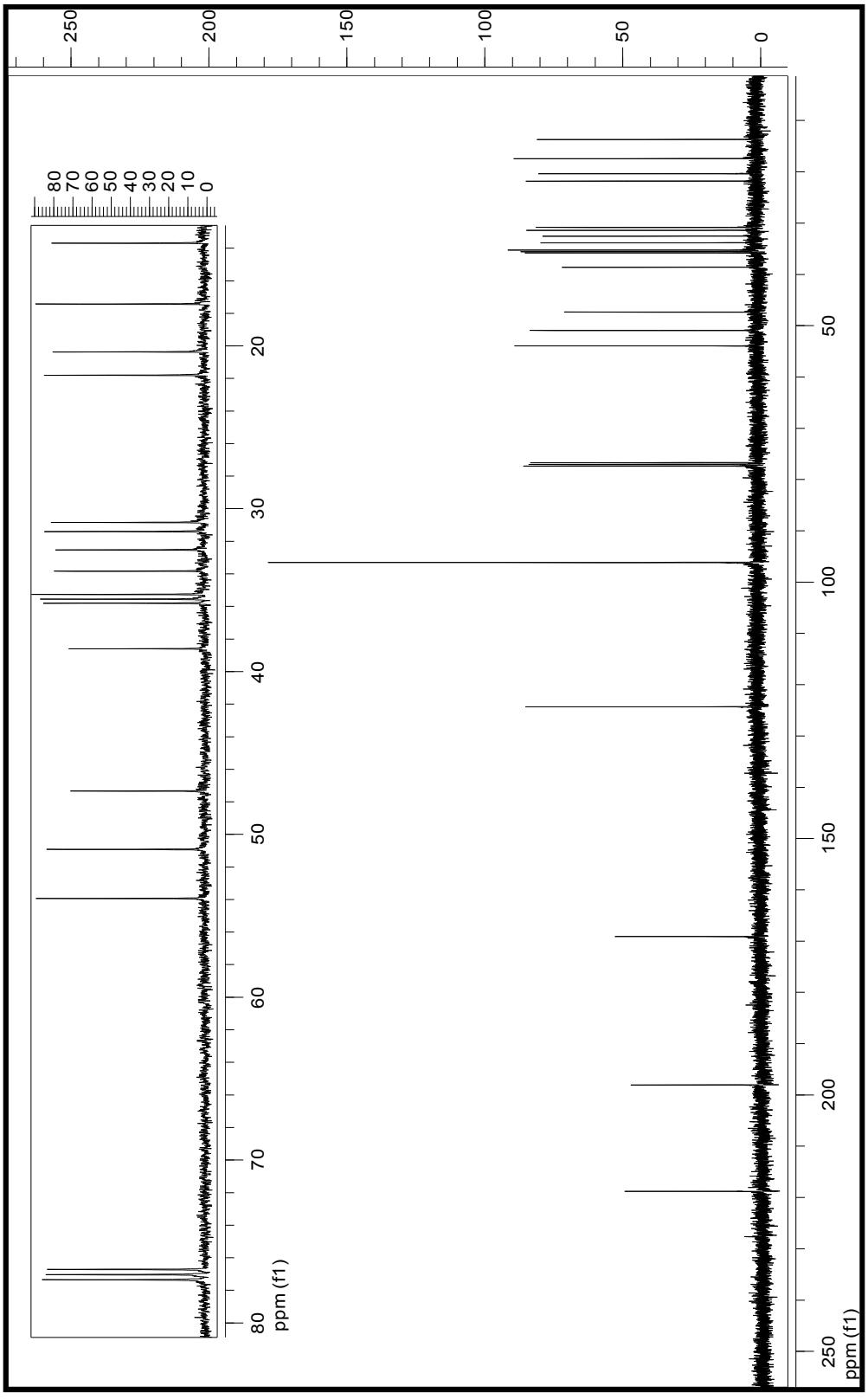


Figure 41. ^{13}C NMR spectrum of starting material, 4-androstene-3,17-dione

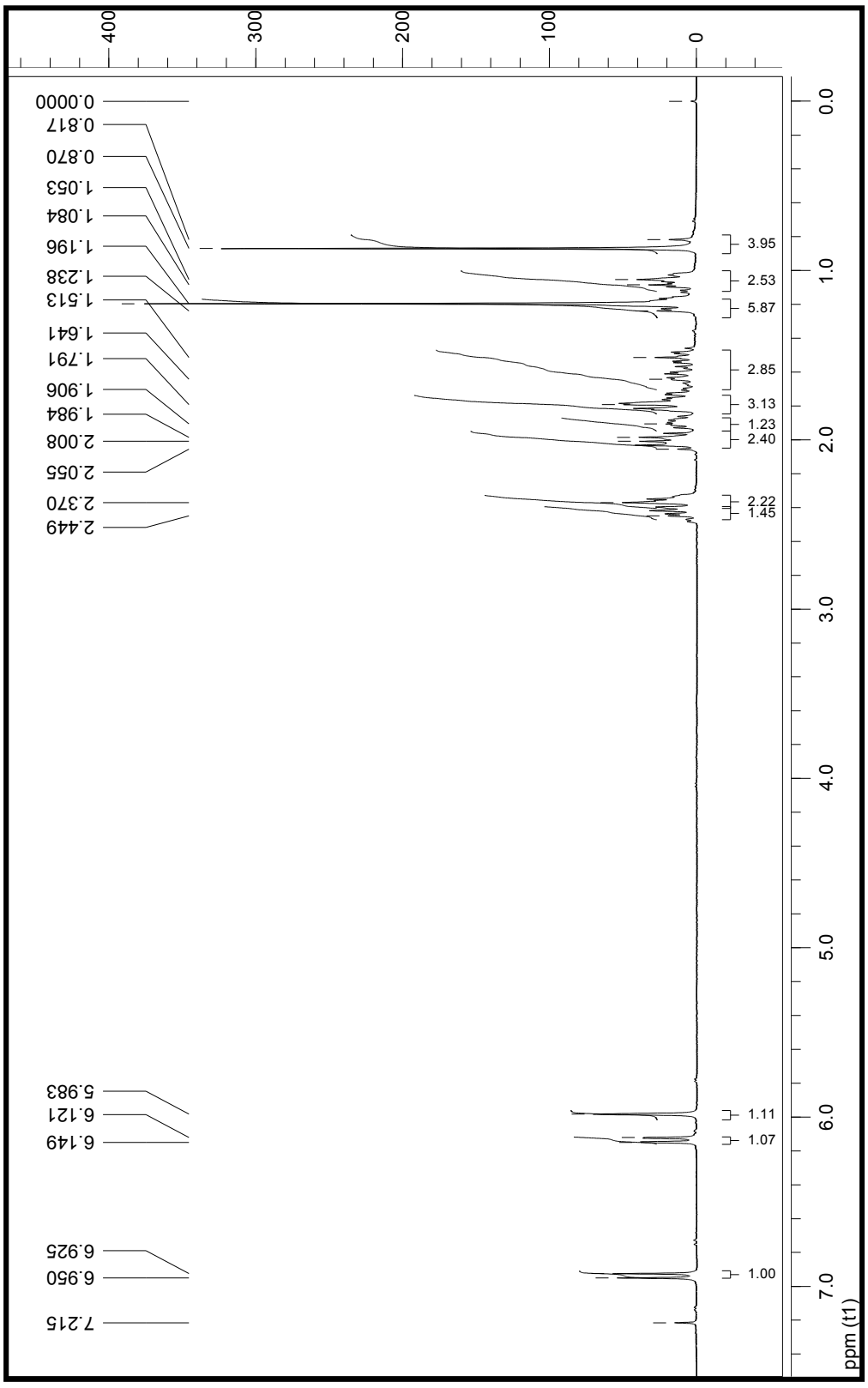


Figure 42. ^{1}H NMR spectrum of androsta-1,4-dien-3,17-dione

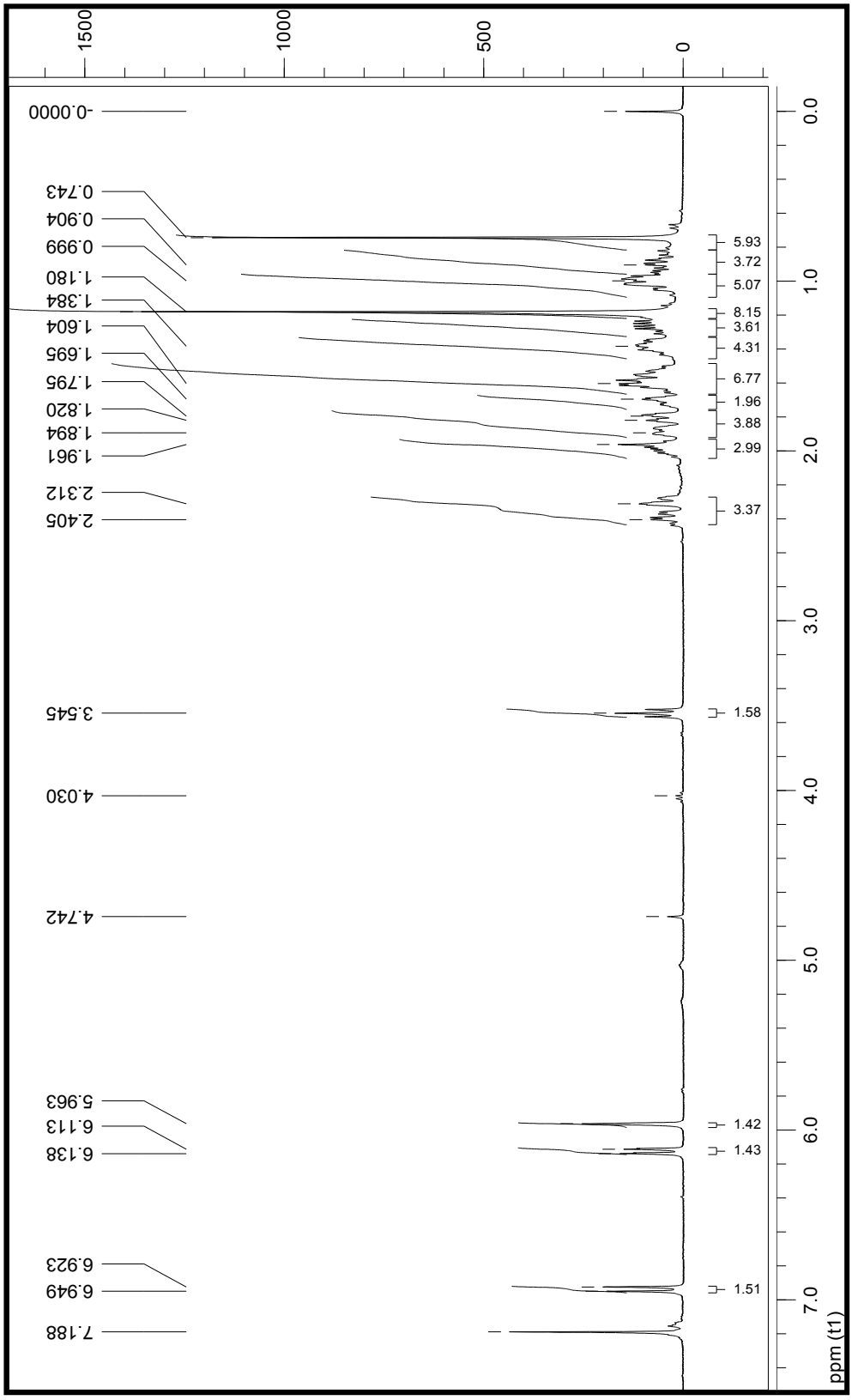


Figure 43. ^{1}H NMR spectrum of 17-hydroxy-androsta-1,4-diene-3-one

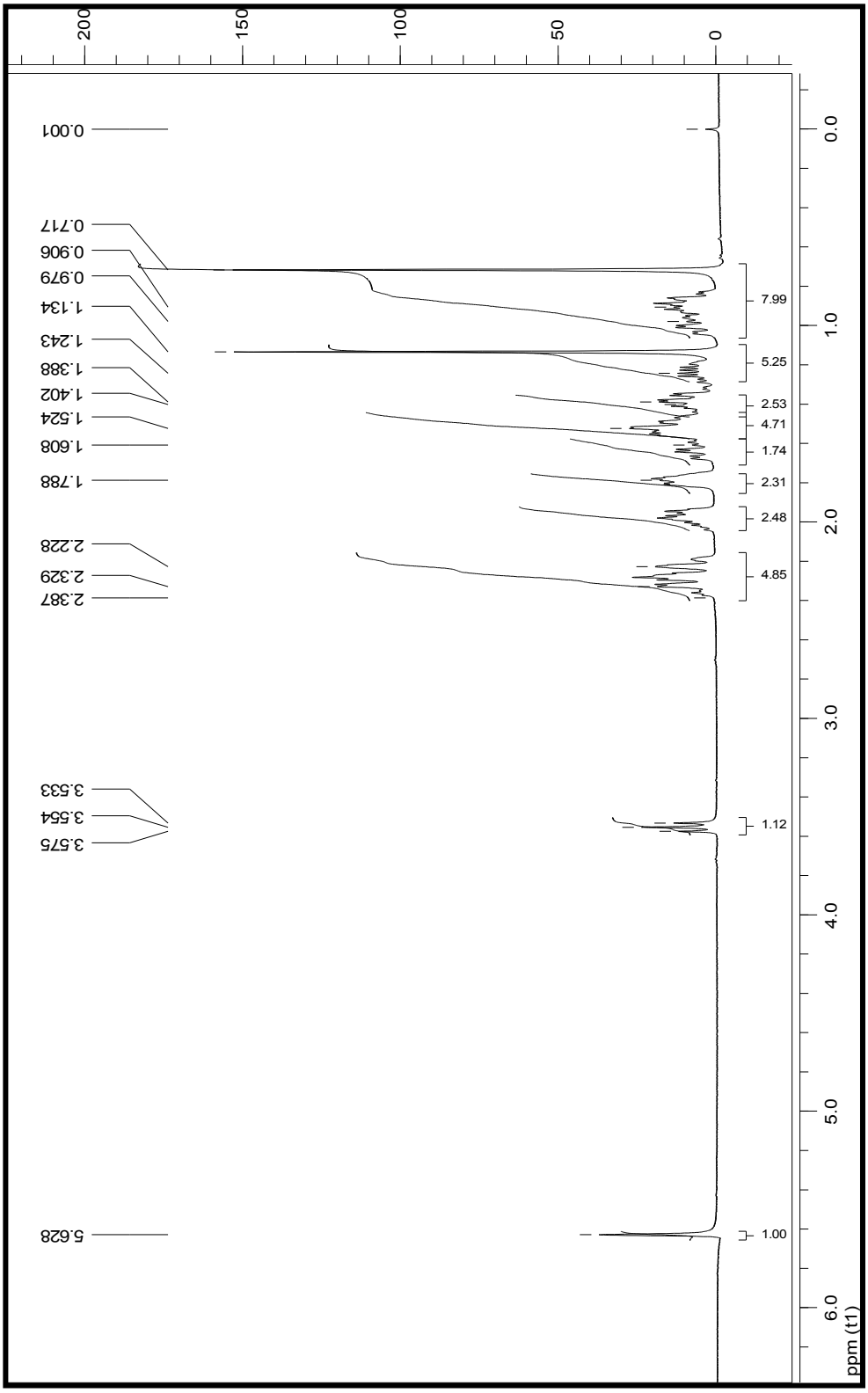


Figure 44. ^{1}H NMR spectrum of 17-hydroxy-androst-4-en-3-one

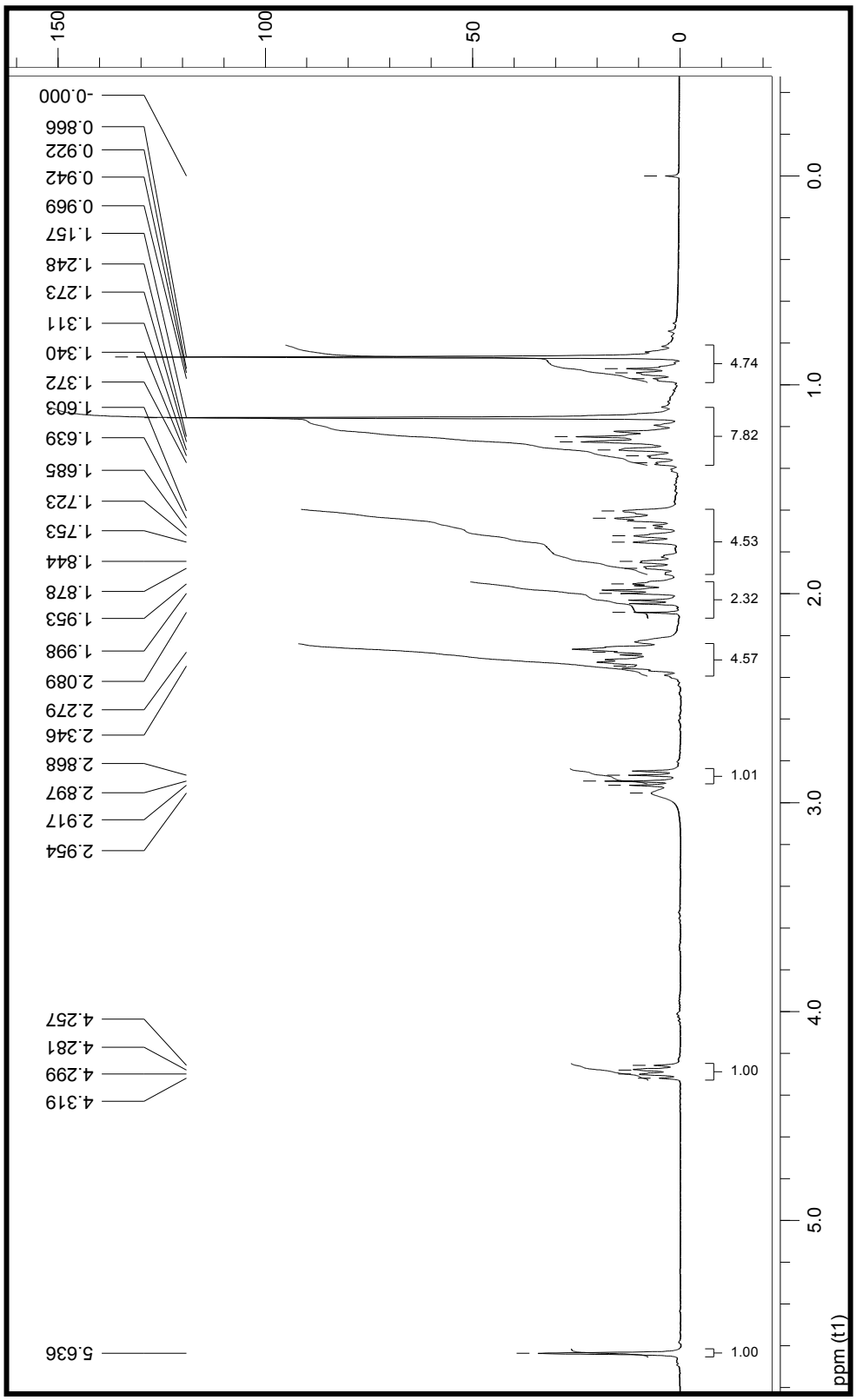


Figure 45. ^{1}H NMR spectrum of androst-4-ene-3,17-diol

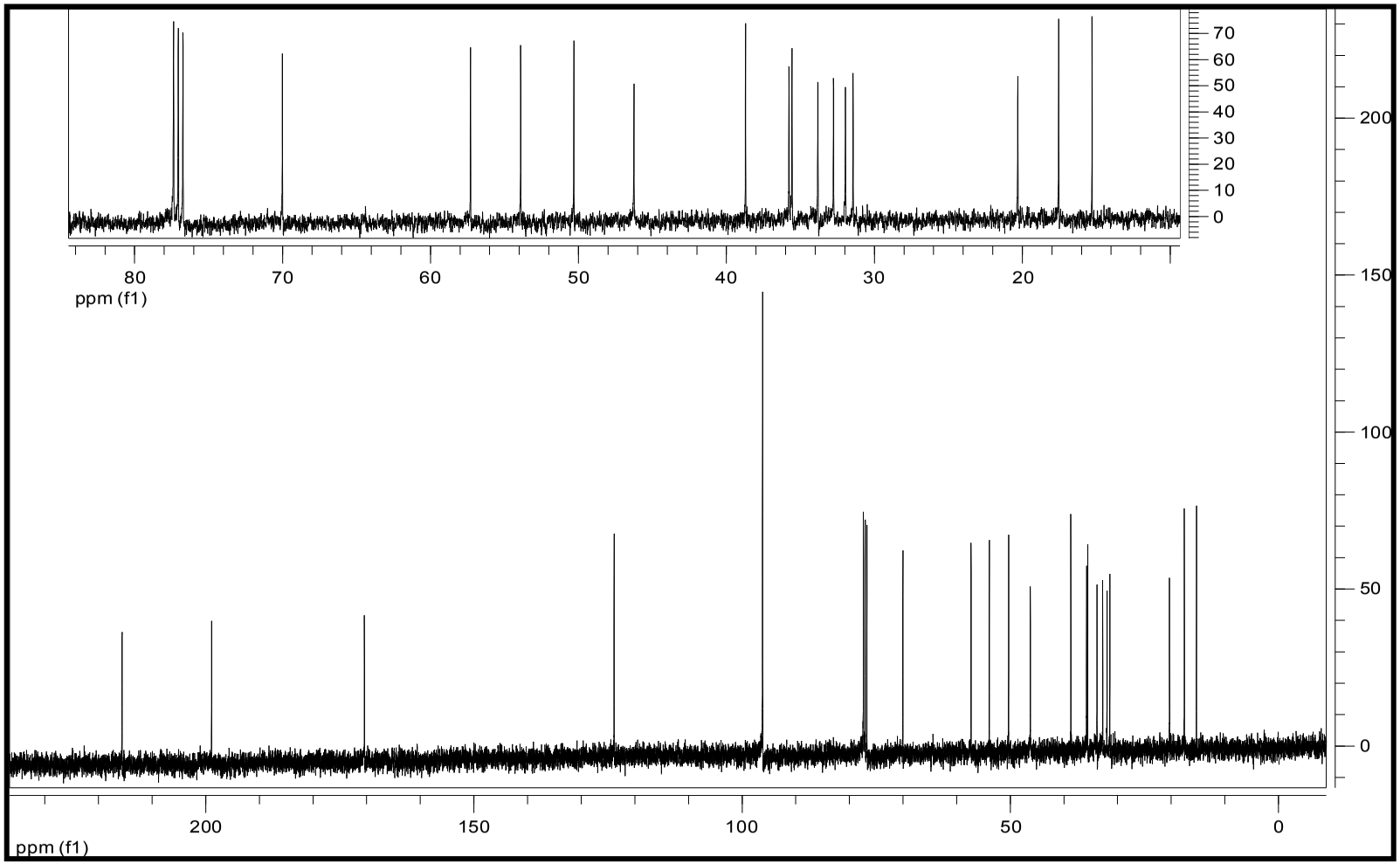


Figure 46. ^{13}C NMR spectrum of androst-4-ene-3,17-diol